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(54) Title: CLONING AND CHARACTERIZING OF GENES ASSOCIATED WITH LONG-TERM MEMORY
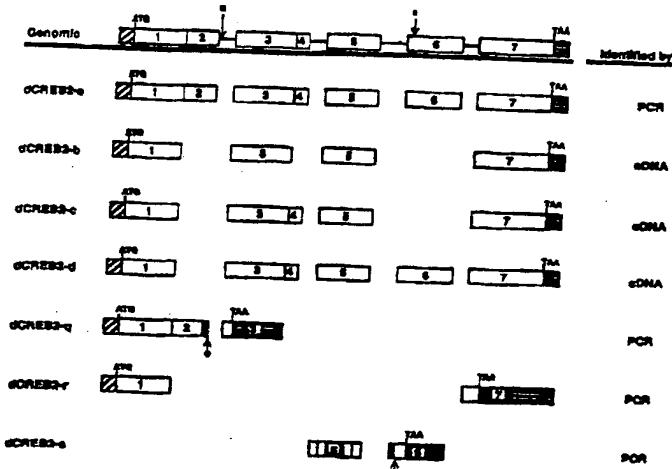
(57) Abstract

A method of regulating long-term memory is disclosed. Also disclosed is isolated DNA encoding a cyclic 3', 5'-adenosine monophosphate responsive transcriptional activator, isolated DNA encoding a' antagonist of cyclic 3', 5'-adenosine monophosphate-inducible transcription, isolated DNA encoding an enhancer-specific activator, and isolated DNA encoding a nitric oxide synthase. A method for assessing the effect of a drug on long-term memory formation is also disclosed.

CLONING AND CHARACTERIZING OF GENES ASSOCIATED WITH LONG-TERM MEMORY

Description

Background of the Invention

5   Activation of the cyclic 3′,5′-adenosine monophosphate (cAMP) signal transduction pathway can have long-lasting global consequences through its influence on the expression of specific genes. This is true for simple organisms as well as mammals, where many of the known cAMP-responsive

10 genes can have important neural and endocrine roles. Additional information regarding activation of this pathway would be useful, particularly as this activation pertains to the ability of animals to remember activities or events.

Summary of the Invention

15   The present invention is based on Applicants' discovery of the dCREB1 and dCREB2 genes. The present invention is further based on Applicants' discovery that the *Drosophila* CREB2 gene codes for proteins of opposite functions. One isoform (e.g., dCREB2-a) encodes a cyclic

20 3′,5′-adenosine monophosphate (cAMP)-responsive transcriptional activator. Another isoform (e.g., dCREB2-b) codes for an antagonist which blocks the activity of the activator.

When the blocking form is placed under the control of

25 the heat-shock promoter, and transgenic flies are made, a brief shift in temperature induces the synthesis of the blocker in the transgenic fly. This induction of the blocker (also referred to herein as the repressor) specifically disrupts long-term, protein synthesis

30 dependent memory of an odor-avoidance behavioral paradigm.

-2-

As a result of Applicants' discovery, a method is
herein provided to regulate long term memory in an animal.
The method of regulating long term memory described herein
comprises inducing expression of a dCREB2 gene or a
5   fragment thereof in the animal.

The dCREB2 gene encodes several isoforms. Examples of
an isoform encoded by the dCREB2 gene are dCREB2-a, dCREB2-
b, dCREB2-c, dCREB2-d, dCREB2-q, dCREB2-r and dCREB2-s.

The isoforms encoded by the dCREB2 gene include cAMP-
10 responsive activator isoforms and antagonistic blocker (or
repressor) isoforms of the activator isoforms. Cyclic AMP
responsive activator isoforms can function as a cAMP-
responsive activator of transcription. Antagonistic
repressors can act as a blocker of activators. An example
15 of a cAMP-responsive activator isoform is dCREB2-a. An
example of an antagonistic repressor (or blocker) isoform
is dCREB2-b. The terms blocker and repressor are used
interchangeably herein.

In one embodiment of the invention, the dCREB-2 gene
20 encodes a cAMP-responsive activator isoform and inducing
said gene results in the potentiation of long term memory.

Alternatively, inducing the dCREB2 gene encoding a
cAMP-responsive activator isoform activates the production
of a protein which is necessary for the formation of long
25 term memory.

In another embodiment of the invention, the dCREB2
gene encodes a repressor isoform and inducing said gene
results in the blocking of long term memory.

A further embodiment of the invention relates to a
30 method of regulating long term memory in an animal
comprising inducing repressor and activator isoforms of
dCREB2 wherein long term memory is potentiated in the
animal when the net amount of functional activator (ΔC) is
greater than zero.

-3-

The invention also relates to a method of identifying a substance capable of affecting long term memory in an animal comprising the determination that said substance alters the induction or activity of repressor and activator
5   isoforms of dCREB2 from normal in the animal.

As referred to herein, an activator isoform includes dCREB2-a and functional fragments thereof and a repressor isoform includes dCREB2-b and functional fragments thereof.

Other embodiments of the invention relate to a method
10   of enhancing long term memory formation in an animal comprising increasing the level of activator homodimer from normal, decreasing the level of activator-repressor heterodimer from normal, or decreasing the level of repressor homodimer from normal in the animal.

15   Still another embodiment of the invention relates to a method of identifying a substance capable of affecting long term memory in an animal comprising the determination that said substance alters activator homodimer, activator-repressor heterodimer and/or repressor homodimer formation
20   from normal in the animal.

As referred to herein, an activator homodimer includes the dCREB2a homodimer, an activator-repressor heterodimer includes the dCREB2a-dCREB2b heterodimer, and a repressor homodimer includes the dCREB2b homodimer.

25   A further embodiment of the invention relates to isolated DNA encoding a cAMP responsive transcriptional activator. Such a cAMP responsive transcriptional activator can be encoded by a *Drosophila* dCREB2 gene or by homologues or functional fragments thereof. For example, a
30   cAMP responsive transcriptional activator can be encoded by the dCREB2 gene which codes for dCREB2-a or by a gene encoded by the sequences presented herein.

Still another embodiment of the invention relates to isolated DNA encoding an antagonist of cAMP-inducible
35   transcription. Such an antagonist of cAMP-inducible

-4-

transcription can be encoded by a *Drosophila* dCREB2 gene or by homologues or functional fragments thereof. For example, an antagonist of cAMP-inducible transcription can be encoded by the dCREB2 gene which codes for dCREB2-b.

5   Another embodiment of the invention relates to isolated DNA (SEQ ID NO.: 25) which encodes a *Drosophila* dCREB2 gene or functional fragments thereof.

A further embodiment of the invention relates to isolated DNA encoding an enhancer-specific activator. Such an enhancer-specific activator can be encoded by a

10   *Drosophila* dCREB1 gene or by homologues or functional fragments thereof.

Another embodiment of the invention relates to isolated DNA encoding a nitric oxide synthase of *Drosophila*

15   (DNOS). Such DNA can encode a DNOS of neuronal locus. The DNOS encoded can contain, for example, putative heme, calmodulin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate, in its reduced form, (NADPH) binding site

20   domains.

A further embodiment of the invention relates to a method for assessing the effect of a drug on long term memory formation comprising administering the drug to *Drosophila*, subjecting the *Drosophila* to classical

25   conditioning to at least one odorant and electrical shock, and assessing the performance index of the classical conditioning, wherein the effect of the drug occurs when it alters the performance index from normal. The drug can affect long term memory formation by, for example, altering

30   the induction or activity of repressor and activator isoforms of dCREB2.

A still further embodiment of the invention relates to the assessment that an animal will have an enhanced or, alternatively, a diminished capability of possessing long

35   term memory. This assessment can be performed by

-5-

determining the amount of cAMP-responsive activator
isoforms, cAMP-responsive repressor or blocker isoforms, or
dimers of these isoforms that are present in the animal,
where these isoforms are encoded by the CREB2 or a

5   homologous gene. Enhanced capability of possessing long
term memory will be more likely as the amount of activator
exceeds the amount of repressor, i.e. in direct proportion
to the size of the net amount of functional activator (ΔC)
when this quantity is greater than zero. Conversely,

10   diminished capability of processing long term memory will
be more likely as the amount of repressor exceeds the
amount of activator, i.e. in direct proportion to the size
of the net amount of functional activator (ΔC) when this
quantity is less than zero.

15   Another embodiment of the invention relates to a
screening assay of pharmaceutical agents as enhancers of
long term memory or as obstructors of long term memory in
animals. The screening assay is performed by determining
the change in the amount of cAMP-responsive activator

20   isoforms, cAMP-responsive repressor or blocker isoforms, or
dimers of these isoforms that is present in an animal or,
more preferably, in a cell culture system or in Drosophila
when the pharmaceutical agent is present, in comparison to
when the pharmaceutical agent is not present, where these

25   isoforms are encoded by the CREB2 or a homologous gene.
Enhancers of long term memory cause a net increase in the
amount of activator isoforms relative to the amount of
repressor isoforms, i.e. an increase in the net amount of
functional activator (ΔC). Obstructors of long term memory

30   cause a net decrease in the amount of activator isoforms
relative to the amount of repressor isoforms, i.e. a
decrease in the net amount of functional activator (ΔC).
The pharmaceutical agent can cause these changes by acting,
for example, to alter the expression (transcription or

35   translation) of the respective activator and/or repressor

-6-

isoforms from the CREB2 or a homologous gene, to alter the
formation of activator homodimers, activator-repressor
heterodimers and/or repressor homodimers from the expressed
isoforms, or to alter the interaction of one or more of
5   these isoform or dimer types at their molecular targets.
The long term memory activator isoform/repressor isoform
system herein disclosed provides a unique platform for
conducting such screening assays.

A further embodiment of the invention relates to an
10   assay of pharmaceutical agents for their property as
facilitators or hinderers of long term memory in animals.
The assay is performed by administering the pharmaceutical
agent to Drosophila prior to subjecting the Drosophila to a
Pavlovian olfactory learning regimen. This regimen
15   assesses the long term memory capabilities of the
Drosophila by subjecting the flies to a massed and/or a
spaced training schedule. Transgenic lines of these flies
containing altered dCREB2 genes can be used to further
elucidate the long term memory facilitation or hindering
20   property of the pharmaceutical agent. The assay provides
data regarding the acquisition of long term memory by the
Drosophila after exposure to the pharmaceutical agent.
These data are compared to long term memory acquisition
data from Drosophila that have not been exposed to the
25   pharmaceutical agent. If the exposed flies display faster
or better retained long term memory acquisition than the
unexposed flies, the pharmaceutical agent can be considered
to be a facilitator of long term memory. Conversely, if
the exposed flies display slower or less retained long term
30   memory acquisition than the unexposed flies, the
pharmaceutical agent can be considered to be a hinderer of
long term memory. Since the genetic locus for this long
term memory assay in Drosophila resides in the dCREB2 gene,
the results from this assay can be directly applied to

-7-

other animals that have homologous genetic loci (CREB2 or CREM genes).

Brief Description of the Drawings

Figure 1A depicts the DNA sequence (SEQ ID NO.: 1) and
5   predicted amino acid sequence (SEQ ID NO.: 2) of the
dCREB2-a coding region. The basic region and leucine
zipper domains are indicated by solid and broken bold
underlining, respectively; positively-charged residues in
the basic region are circled; periodic leucines in the
10 zipper motif are boxed; glutamines in the activation domain
are underlined; the short amino acid motif with target
sites for kinases, starting at residue 227, is indicated by
a bold outline; and sequences specified by alternatively-
spliced exons 2, 4 and 6 are shaded.
15   Figure 1B depicts the amino acid sequences of the bZIP
domains of dCREB2 (SEQ ID NO.: 3), mammalian CREB (SEQ ID
NO.: 4), CREM (SEQ ID NO.: 5) and ATF-1 (SEQ ID NO.: 6).
Differences between dCREB2 and CREB are boxed.
Figure 2 is a schematic diagram of dCREB2 isoforms
20 with the exon boundaries defined with respect to dCREB2-a.
Diagram is not drawn to scale.
Figure 3 is a bar graph representation of results
showing pKA-responsive transcriptional activation by
dCREB2-a.
25   Figure 4 is a bar graph representation of results
showing the transcriptional effect of dCREB2-b and a mutant
variant on pKA-responsive activation by dCREB2-a.
Figure 5 depicts the DNA sequence (SEQ ID NO.: 7) and
predicted amino acid sequence (SEQ ID NO.: 8) of the dCREB1
30 coding region. The basic region and leucine zipper domains
are indicated by solid and broken bold underlining,
respectively; positively-charged residues in the basic
region are circled; periodic leucines of the zipper motif
are boxed; and in the acid-rich region of the activation

domain, negatively-charged amino acids are underlined and proline residues are indicated by diamonds.

Figure 6 is a bar graph representation of results showing transcriptional activation of a CRE reporter gene
5   by dCREB1 in Drosophila Schneider L2 cell culture.

Figure 7A is a photomicrograph of a Northern blot depicting the effect of heat shock induction on dCREB2-b expression: wt = wildtype flies; CREB = 17-2 transgenic flies; lanes 1-2: no heat shock; lanes 2-3: immediately
10   after heat shock; lanes 5-6: three hours after heat shock.

Figure 7B is a photograph of a Western blot depicting the effect of heat shock induction on dCREB2-b protein production: wt = wildtype flies; CREB = 17-2 transgenic flies; lanes 1-2: no heat shock; lanes 2-3: immediately
15   after heat shock; lanes 5-6: one hour after heat shock; lanes 7-8: three hours after heat shock; lanes 9-10: 9 hours after heat shock; lanes 11-12: 24 hours after heat shock.

Figure 7C is a photograph of a Western blot depicting
20   the effect of heat shock induction on dCREB2 and dCREB2-mLZ (a mutated dCREB2-b) protein production: wt = 17-2 transgenic flies (expressing wildtype blocker, dCREB2-b); m = A2-2 transgenic flies (expressing mutant blocker, dCREB2-mLZ); lanes 1-2: no heat shock; lanes 3-4: immediately
25   after heat shock; lanes 5-6: three hours after heat shock; lanes 7-8: six hours after heat shock.

Figure 8 is a bar graph representation of results showing the effect of cycloheximide (CXM) feeding, before or after spaced or massed training, on one-day memory
30   retention: stripped bars = +CXM; hatched bars = -CXM.

Figure 9A is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype (Can-S) flies and hs-dCREB2-b transgenic (17-2) flies given spaced or massed training:

-9-

hatched bars = wildtype (Can-S) flies; stripped bars: *hs-dCREB2-b* transgenic (17-2) flies; hs = heat shock.

Figure 9B is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype (Can-S) flies or *hs-dCREB2-b* transgenic (M11-1) flies given spaced or massed training: hatched bars = wildtype (Can-S) flies; stripped bars: *hs-dCREB2-b* transgenic (M11-1) flies; hs = heat shock.

Figure 9C is a bar graph representation of results showing the effect of heat shock induction on learning in wildtype (Can-S) flies and *hs-dCREB2-b* transgenic (17-2) flies given spaced or massed training: hatched bars = wildtype (Can-S) flies; stripped bars: *hs-dCREB2-b* transgenic (17-2) flies; hs = heat shock.

Figure 10 is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in wildtype [*w(isoCJ1)*] flies, *hs-dCREB2-b* transgenic (17-2) flies, and mutant *hs-dCREB2-mLZ* transgenic (A2-2) flies given spaced training: hatched bars = wildtype [*w(isoCJ1)*] flies; stripped bars = *hs-dCREB2-b* transgenic (17-2) flies; white bars = mutant *hs-dCREB2-mLZ* transgenic (A2-2) flies; hs = heat shock.

Figure 11 is a bar graph representation of results showing the effect of heat shock induction on seven-day memory retention (long term memory) in wildtype (Can-S) flies and *hs-dCREB2-b* transgenic (17-2) flies given spaced training: hatched bars = wildtype (Can-S) flies; stripped bars = *hs-dCREB2-b* transgenic (17-2) flies; hs = heat shock.

Figure 12 is a bar graph representation of results showing the effect of heat shock induction on one-day memory retention in *hs-dCREB2-b* transgenic (17-2) flies, *radish* mutant flies, and *radish hs-dCREB2-b* double mutant (*rsh;17-2*) flies given spaced training: hs = heat shock; hatched bars = -hs; stripped bars = +hs.

-10-

Figure 13A is a graphic representation of results showing the effect of repeated training sessions on seven-day memory retention (long term memory) in wildtype (Can-S) flies with long term memory as a function of the number of
5   training sessions indicated by open circles and a negative accelerating exponential Gompertz (growth) function fit to the individual performance indexes (PIs) using a nonlinear iterative least squares method indicated by the solid line.

Figure 13B is a graphic representation of results
10 showing the effect of the rest interval between each training session on seven-day memory retention (long term memory) in wildtype (Can-S) flies with long term memory as a function of the rest interval indicated by open circles and a negative accelerating exponential Gompertz (growth)
15 function fit to the individual performance indexes (PIs) using a nonlinear iterative least squares method indicated by the solid line.

Figure 14 depicts a conceptual model of a molecular switch for the formation of long term memory based on
20 differential regulation of CREB isoforms with opposing functions with ΔC indicating the net effect of CREB activators.

Figure 15A is a bar graph representation of results showing the effect of 48 massed training sessions (48x
25 massed) or 10 spaced training sessions with a 15-minute rest interval (10x spaced) on seven-day memory retention in wildtype (Can-S) flies.

Figure 15B is a bar graph representation of results showing the effect of one (1x), two (2x) or ten (10x)
30 massed training sessions, three hours after heat-shock induction of the transgene (induced) or in the absence of heat-shock (uninduced), on seven-day memory retention in wildtype (Can-S) flies, hsp-dCREB2-a transgenic (C28) flies, and hsp-dCREB2-a transgenic (C30) flies: black bars
35 = wildtype (Can-S) flies; stripped bars = hsp-dCREB2-a

-11-

transgenic (C28) flies; and white bars = *hsp-dCREB2-a*
transgenic (C30) flies.

Figure 15C is a bar graph representation of results
showing responses three hours after heat shock in wildtype
5   (Can-S) flies and *hsp-dCREB2-a* transgenic (*C28*) flies to
odors, either octanol (OCT) or methylcyclohexanol (MCH), or
to shock (60 V DC): black bars = wildtype (Can-S) flies;
stripped bars = *hsp-dCREB2-a* transgenic (C28) flies.

Figure 16A-16C depict the deduced amino acid sequences
10 of DNOS and mammalian NOSs with amino acid numbering
starting at the first methionine in each open reading frame
(ORF), putative binding domains for cofactors (overlined)
demarcated as in previously published reports on mammalian
NOSs, and amino acids which have been proposed as contacts
15 with FAD and NADPH based on crystal structure of the
ferrodoxin NADP⁺ reductase (Karplus, P.A., *Science, 251*:
60-66 (1991)) conserved at equivalent positions (bullet
points): DNOS, *Drosophila* NOS (SEQ ID NO.: 9); RNNOS, rat
neuronal NOS (SEQ ID NO.: 10); BENOS, bovine endothelial
20 NOS (SEQ ID NO.: 11); MMNOS, mouse macrophage NOS (SEQ ID
NO.: 12). Sequence alignment and secondary structure
predictions were performed by Geneworks 2.3
(IntelliGenetics).

Figure 16D is a schematic diagram of the domain
25 structure of *Drosophila* and mammalian NOS proteins with the
proposed cofactor-binding sites for heme (H), calmodulin
(CaM), flavin mononucleotide (FMN), flavin adenine
dinucleotide (FAD), nicotinamide adenine dinucleotide
phosphate (NADPH) and the glutamine-rich domain (Q) in DNOS
30 shown.

Figure 17A is a photograph of a Western blot showing
DNOS expression in 293 human embryonic kidney cells.

Figure 17B is a bar graph representation of results
showing DNOS enzyme activity measured in 293 human
35 embryonic kidney cell extracts by conversion of ³H-L-

-12-

arginine to ³H-L-citrulline: in the presence of exogenous
Ca²⁺ or calmodulin (group B); in the presence of 1 mM EGTA
without exogenous Ca²⁺ or calmodulin (group C); in the
presence of 100 mM L-NAME with exogenous Ca²⁺ or calmodulin
5   (group D).

        Figure 18A is a photomicrograph of a Northern blot
showing a 5.0 kb dNOS transcipt present in Drosophila
heads: H = head; B = body.

        Figure 18B is a photograph of an agarose gel stained
10 with ethidium bromide showing the expression by the dNOS
gene of two alternatively spliced mRNA species with the
arrows indicating the positions of the DNA fragments of the
expected sizes: the 444 bp long-form fragment and the 129
bp short-form fragment. The other bands present in the
15 lane are artifacts from heteroduplexes that failed to
denature. KB = size markers.

        Figure 18C depicts the alignment of the deduced amino
acid sequence of two protein isoforms of DNOS and mouse
neuronal NOS: top part shows the relation between two
20 conceptual Drosophila NOS proteins, DNOS-1 (amino acid
residues 408-427 and 513-532 of SEQ ID NO.: 9) and DNOS-2
(SEQ ID NO.: 14), corresponding to the longer and shorter
RT-PCR products, respectively; the bottom part shows the
relationship between the relevant regions of two protein
25 isoforms of the mouse neuronal NOS, n-NOS-1 (amino acid
residues 494-513 and 599-618; SEQ ID NO.: 13 and SEQ ID
NO.: 15, respectively) and n-NOS-2 (SEQ ID NO.: 16); and
the numbers indicate the positions of the amino acid
residues relative to the first methionine in the respective
30 OFRs.

        Figure 19A-19B depicts the nucleotide sequence (SEQ ID
NO.: 25) of a dNOS cDNA encoding the DNOS protein. The
open reading frame of 4050 bp starts at nucleotide 189 and
ends at nucleotide 4248.

-13-

Detailed Description of the Invention

Applicants have cloned and characterized two genes,
designated dCREB2 and dCREB1, isolated through a DNA-
binding expression screen of a Drosophila head cDNA library
5   in which a probe containing three cAMP-responsive element
(CRE) sites was used.

The dCREB2 gene codes for the first known cAMP-
dependent protein kinase (PKA) responsive CREB/ATF
transcriptional activator in Drosophila. A protein data
10 base search showed mammalian CREB, CREM and ATF-1 gene
products as homologous to dCREB2. For these reasons,
dCREB2 is considered to be a member, not only of the
CREB/ATF family, but of the specific cAMP-responsive
CREB/CREM/ATF-1 subfamily. It is reasonable to expect that
15 dCREB2 is involved in Drosophila processes which are
analogous to those which are thought to depend on cAMP-
responsive transcriptional activation in other animal
systems.

Applicants have shown that the dCREB2 transcript
20 undergoes alternative splicing. Splice products of dCREB2
were found to fall into two broad categories: one class of
transcripts (dCREB2-a, -b, -c, -d) which employs
alternative splicing of exons 2, 4 and 6 to produce
isoforms whose protein products all have the bZIP domains
25 attached to different versions of the activation domain and
a second class of transcripts (dCREB2-q, -r, -s) which have
splice sites which result in in-frame stop codons at
various positions upstream of the bZIP domain. These all
predict truncated activation domains without dimerization
30 or DNA binary activity.

dCREB2-a,-b,-c and -d are splice forms that predict
variants of the activation domain attached to a common
basic region-leucine zipper. These alternative splice
forms result in seemingly minor changes in the size and
35 spacing of parts of the activation domain. Nevertheless,

-14-

alternative splicing of the activation domain has profound
effects on the functional properties of dCREB2 products.
Isoform dCREB2-a produces a PKA-responsive transcriptional
activator in cell culture, whereas dCREB2-b, lacking exons

5   2 and 6, produces a specific antagonist. This dCREB2
splicing pattern (and its functional consequences) is
virtually identical to that seen in the CREM gene.
Similarly located, alternatively-spliced exons in the CREM
gene determine whether a particular isoform is an activator

10   or an antagonist (deGroot, R.P. and P. Sassone-Corsi, Mol.
Endocrinol., 7: 145-153 (1993); Foulkes, N.S. et al.,
Nature, 355: 80-84 (1992)).

     The ability of the phosphorylation domain (KID domain)
to activate in trans other constitutive transcription

15   factors which are bound nearby could potentially transform
a CREM antagonist (which contains the KID domain but is
lacking an exon needed for activation) into a cAMP-
responsive activator. Since the modular organization of
these molecules has been conserved, dCREB2-d could have

20   this property.

     In contrast to the dCREB2 splicing variants that
encode isoforms with a basic region-leucine zipper domain,
the dCREB2-q, -r and -s splice forms incorporate in-frame
stop codons whose predicted protein products are truncated

25   before the bZIP region. Isoforms of this type have been
identified among the products of the CREB gene (deGroot,
R.P. and P. Sassone-Corsi, Mol. Endocrinol., 7: 145-153
(1993); Ruppert, S. et al., EMBO J., 11: 1503-1512 (1992))
but not the CREM gene. The function of these truncated

30   CREB molecules is not known, but at least one such CREB
mRNA is cyclically regulated in rat spermatogenesis
(Waeber, G. et al., Mol. Endocrinol., 5: 1418-1430 (1991)).

     So far, dCREB2 is the only cAMP-responsive CREB
transcription factor isolated from Drosophila. Other

35   Drosophila CREB molecules, BBF-2/dCREB-A (Abel, T. et al.,

-15-

Genes Dev., *6*: 466-488 (1992); Smolik, S.M. *et al.*, *Mol. Cell Biol.*, *12*: 4123-4131 (1992)), *dCREB-B* (Usui, T. *et al.*, *DNA and Cell Biology*, *12(7)*: 589-595 (1993)) and *dCREB1*, have less homology to mammalian CREB and CREM. It
5   may be that *dCREB2* subsumes functions of both the CREB and CREM genes in *Drosophila*. The mammalian CREB and CREM genes are remarkably similar to one another in several respects. It has been suggested that CREB and CREM are the product of a gene duplication event (Liu, F. *et al.*, *J.*
10 *Biol. Chem.*, *268*: 6714-6720 (1993); Riabowol, K.T. *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, *1*: 85-90 (1988)). *dCREB2* has a striking degree of amino acid sequence similarity to the CREB and CREM genes in the bZIP domain. Moreover, comparison of alternative splicing patterns among
15 CREB, CREM and *dCREB2* indicates that *dCREB2* generates mRNA splicing isoforms similar to exclusive products of both CREB and CREM. Taken together, the sequence information and the splicing organization suggest that *dCREB2* is an ancestor of both the mammalian CREB and CREM genes.
20   As discussed further herein, one phenomenon in which *dCREB2* might act with enduring consequences is in long-term memory. This possibility is a particularly tempting one because recent work in *Aplysia* indicates that a CREB factor is likely to function in long-term facilitation by inducing
25 an "immediate early" gene (Alberini, C.M. *et al.*, *Cell, 76*: 1099-1114 (1994); Dash, P.K., *Nature, 345*: 718-721 (1990)). Recent experiments with a conditionally-expressed *dCREB2-b* transgene indicate that it has specific effects on long-term memory in *Drosophila*.
30   The product of the second gene described herein, *dCREB1*, also appears to be a member of the CREB/ATF family. Gel-retardation assays indicate that it binds specifically to CREs. It has a basic region and an adjacent leucine zipper at its carboxyl end, but this domain shows limited
35 amino acid sequence similarity to other CREB/ATF genes.

-16-

The presumed transcriptional activation domain of *dCREB1* is
of the acid-rich variety. Furthermore, it has no consensus
phosphorylation site for PKA. *dCREB1* can mediate
transcriptional activation from CRE-containing reporters in
5   the *Drosophila* L2 cell line, but this activation is not
dependent on PKA.

A recurrent finding from work on the biology of
learning and memory is the central involvement of the cAMP
signal transduction pathway. In Aplysia, the cAMP
10 second-messenger system is critically involved in neural
events underlying both associative and non-associative
modulation of a behavioral reflex (Kandel, E.R. and J.H.
Schwartz, *Science*, *218*: 433-443 (1982); Kandel, E.R., *et
al.*, *In Synaptic Function*, Edelmann, G.M., *et al.* (Eds.),
15 John Wiley and Sons, New York (1987); Byrne, J.H., *et al.*,
*In Advances in Second Messenger and Phosphoprotein
Research*, Shenolikar, S. and A.C. Nairn (Eds.), Raven
Press, New York, pp. 47-107 (1993)). In *Drosophila*, two
mutants, *dunce* and *rutabaga*, were isolated in a behavioral
20 screen for defects in associative learning and are lesioned
in genes directly involved in cAMP metabolism (Quinn, W.G.,
et al., *Proc. Natl. Acad. Sci. USA*, *71*: 708-712 (1974);
Dudai, Y., *et al.*, *Proc. Natl. Acad. Sci.*, *USA 73*: 1684-
1688 (1976); Byers, D. *et al.*, *Nature*, *289*: 79-81 (1981);
25 Livingstone, M.S., *et al.*, *Cell*, *37*: 205-215 (1984); Chen,
C.N. et al., *Proc. Natl. Acad. Sci. USA*, *83*: 9313-9317
(1986); Levin, L.R., *et al.*, *Cell*, *68*: 479-489 (1992)).
These latter observations were extended with a
reverse-genetic approach using inducible transgenes
30 expressing peptide inhibitors of cAMP-dependent protein
kinase (PKA) and with analyses of mutants in the PKA
catalytic subunit (Drain, P. *et al.*, *Neuron*, *6*: 71-82
(1991); Skoulakis, E.M., *et al.*, *Neuron*, *11*: 197-208
(1993)). Recent work on mammalian long-term potentiation
35 (LTP) also has indicated a role for cAMP in synaptic

-17-

plasticity (Frey, U., et al., *Science, 260*: 1661-1664
(1993); Huang, Y.Y. and E.R. Kandel, *In Learning and
Memory*, vol. 1, pp.74-82, Cold Spring Harbor Press, Cold
Spring Harbor, NY (1994)).

5   The formation of long-lasting memory in animals and of
long-term facilitation in Aplysia can be disrupted by drugs
that interfere with transcription or translation (Agranoff,
B.W. et al., *Brain Res., 1*: 303-309 (1966); Barondes, S.H.
and H.D. Cohen, *Nature, 218*: 271-273 (1968); Davis, H.P.

10 and L.R. Squire, *Psychol. Bull., 96*: 518-559 (1984);
Rosenzweig, M.R. and E.L. Bennett, *In Neurobiology of
Learning and Memory*, Lynch, G., et al. (Eds.), The Guilford
Press, New York, pp. 263-288, (1984); Montarolo, P.G., et
al., *Science, 234*: 1249-1254 (1986)). This suggests that

15 memory consolidation requires *de novo* gene expression.
Considered along with the involvement of the cAMP
second-messenger pathway, this requirement for newly
synthesized gene products suggests a role for
cAMP-dependent gene expression in long-term memory (LTM)

20 formation.

     In mammals, a subset of genes from the CREB/ATF family
are known to mediate cAMP-responsive transcription
(Habener, J.F., *Mol. Endocrinol., 4*: 1087-1094 (1990);
deGroot, R.P. and P. Sassone-Corsi, *Mol. Endocrinol., 7*:

25 145-153 (1993)). CREBs are members of the basic region-
leucine zipper transcription factor superfamily;
(Landschulz, W.H. et al., *Science, 240*: 1759-1764 (1988)).
The leucine zipper domain mediates selective homo- and
hetero-dimer formation among family members (Hai, T.Y. et

30 al., *Genes & Dev., 3*: 2083-2090 (1989); Hai, T. and T.
Curran, *Proc. Natl. Acad. Sci. USA, 88*: 3720-3724 (1991)).
CREB dimers bind to a conserved enhancer element (CRE)
found in the upstream control region of many
cAMP-responsive mammalian genes (Yamamoto, K.K., et al.,

35 *Nature, 334*: 494-498 (1988)). Some CREBs become

-18-

transcriptional activators when specifically phosphorylated by PKA (Gonzalez, G.A. and M.R. Montminy, *Cell*, *59*: 675-680 (1989); Foulkes, N.S. *et al.*, *Nature*, *355*: 80-84 (1992)), while others, isoforms from the CREM gene, are functional

5 antagonists of these PKA-responsive activators (Foulkes, N.S. *et al.*, *Cell*, *64*: 739-749 (1991); Foulkes, N. and P. Sassone-Corsi, *Cell*, *68*: 411-414 (1992)).

Work in Aplysia has shown that cAMP-responsive transcription is involved in long-term synaptic plasticity

10 (Schacher, S. *et al.*, *Science*, *240*: 1667-1669 (1988); Dash, P.K., *Nature*, *345*: 718-721 (1990)). A primary neuronal co-culture system has been used to study facilitation of synaptic transmission between sensory and motor neurons comprising the monosynaptic component of the Aplysia

15 gill-withdrawal reflex. Injection of oligonucleotides containing CRE sites into the nucleus of the sensory neuron specifically blocked long-term facilitation (Dash, P.K., *Nature*, *345*: 718-721 (1990)). This result suggests that titration of CREB activity might disrupt long-term synaptic

20 plasticity.

Described herein is the cloning and characterization of a *Drosophila* CREB gene, *dCREB2*. This gene produces several isoforms that share overall structural homology and nearly complete amino acid identity in the basic

25 region-leucine zipper with mammalian CREBs. The dCREB2-a isoform is a PKA-responsive transcriptional activator whereas the dCREB2-b product *blocks* PKA-responsive transcription by dCREB2-a in cell culture. These molecules with opposing activities are similar in function to

30 isoforms of the mammalian CREM gene (Foulkes, N.S. *et al.*, *Cell*, *64*: 739-749 (1991); Foulkes, N. and P. Sassone-Corsi, *Cell*, *68*: 411-414 (1992); Foulkes, N.S. *et al.*, *Nature*, *355*: 80-84 (1992)). The numerous similarities in sequence and function between *dCREB2* and mammalian CREBs suggest

-19-

that cAMP-responsive transcription is evolutionarily conserved.

Genetic studies of memory formation in *Drosophila* have revealed that the formation of a protein synthesis-
5   dependent long-term memory (LTM) requires multiple training sessions with a rest interval between them. As discussed further herein, this LTM is blocked specifically by induced expression of a repressor isoform of the cAMP-responsive transcription factor CREB. Also as discussed further
10   herein, LTM information is enhanced after induced expression of an activator form of CREB. Maximum LTM is achieved after just one training session.

To investigate the role of CREBs in long-term memory (LTM) formation in Drosophila, dominant-negative transgenic
15   lines which express *dCREB2-b* under the control of a heat-shock promoter *(hs-dCREB2-b)* were generated. Groups of flies, which had been heat-shock induced or left uninduced, were tested for memory retention after Pavlovian olfactory learning. This acute induction regimen minimized
20   potential complications from inappropriate expression of dCREB2-b during development and allowed a clear assessment of the effect of *hs-dCREB2-b* induction on memory formation.

In Drosophila, consolidated memory after olfactory learning is composed of two genetically distinct
25   components: anesthesia-resistant memory (ARM) and long-term memory (LTM). ARM decays to zero within four days after training, and formation of ARM is insensitive to the protein synthesis inhibitor cycloheximide (CXM) but is disrupted by the *radish* mutation (Folkers, E., *et al.*,
30   *Proc. Natl. Acad. Sci. USA, 90*: 8123-8127 (1993)). In contrast, LTM shows essentially no decay over at least seven days, its formation is cycloheximide-sensitive and it is not disrupted by *radish*. Two different training protocols involving massed and spaced sessions were
35   employed (Ebbinghaus, H., *Uber das Gedachtnis*, Dover, New

-20-

York (1885); Baddeley, A.D., *The Psychology of Memory*,
Basic Books, New York (1976)) to dissect memory formation.
The massed training procedure consists of ten consecutive
training cycles with no rest interval between them, while
5 the spaced training protocol consists of the same number of
sessions but with a 15-minute rest between each. Their
genetic dissection revealed that the massed protocol
produced only ARM, while the spaced protocol produced
memory retention composed of both ARM and LTM.
10   The behavioral results show that formation of LTM is
completely blocked by induced expression of hs-dCREB2-b.
This effect is remarkable in its behavioral specificity.
ARM, a form of consolidated memory genetically
distinguishable from LTM, but co-existing with it one-day
15 after spaced training, was not affected. Learning and
peripheral behaviors likewise were normal. Thus, the
effect of the induced hs-dCREB2-b transgene is specific to
LTM.
        Induction of the mutant blocker did not affect LTM.
20 This result, together with the molecular data which showed
that induction of the wild-type blocker did not have
widespread effects on transcription, suggests that the
blocker is reasonably specific at the molecular level when
it specifically blocks LTM. The wild-type blocker may
25 disrupt cAMP-dependent transcription *in vivo*, since it can
block cAMP-responsive transcription in cell culture. It is
reasonable to infer that dimerization is necessary for
blocker function and that the wild-type blocker could
interfere with cAMP-responsive transcription either by
30 forming heterodimers with dCREB2-a, the activator, or by
forming homodimers and competing for DNA binding with
homodimers of dCREB2-a. Thus, activators and repressors
may form homodimers or heterodimers. It is reasonable to
infer that long term memory is enhanced when the level of
35 activator homodimer is increased from normal and/or when

-21-

the level of activator-repressor heterodimer is decreased
from normal and/or when the level of repressor homodimer is
decreased from normal. In any case, the molecular
target(s) of dCREB2-b are likely to be interesting because
5 of the behavioral specificity of the block of LTM.

In *Drosophila*, consolidation of memory into long-
lasting forms is subject to disruption by various agents.
A single-gene mutation *radish* and the pharmacological agent
CXM were used to show that long-lasting memory in flies is
10 dissectable into two components, a CXM-insensitive ARM,
which is disrupted by *radish*, and a CXM-sensitive LTM,
which is normal in *radish* mutants. As described herein,
CREB-family members are likely to be involved in the CXM-
sensitve, LTM branch of memory consolidation. The results
15 described herein, taken together with the showing that
long-term memory is dissectable into a CXM-insentive ARM
and a CXM-sensitive LTM, show that only one functional
component of consolidated memory after olfactory learning
lasts longer than four days, requires *de novo* protein
20 synthesis and involves CREB-family members.

Based on work in Aplysia, a model has been proposed to
describe the molecular mechanism(s) underlying the
transition from short-term, protein synthesis-independent
to long-term, protein synthesis-dependent synaptic
25 plasticity (Alberini, C.M. *et al.*, *Cell*, *76*: 1099-1114
(1994)). The present work in Drosophila on long-term
memory extends this model to the whole organism. Important
molecular aspects of this transition seem to involve
migration of the catalytic subunit of PKA into the nucleus
30 (Backsai, B.J. *et al.*, *Science*, *260*: 222-226 (1993)) and
subsequent phosphorylation and activation of CREB-family
members (Dash, P.K., *Nature*, *345*: 718-721 (1990); Kaang,
B.K., *et al.*, *Neuron*, *10*: 427-435 (1993)). In flies, it is
likely that the endogenous dCREB2-a isoform is one of these
35 nuclear targets. Activated dCREB2-a molecules then might

-22-

transcribe other target genes, including the immediate
early genes--as is apparently the case in Aplysia.
(Alberini, C.M. *et al.*, *Cell*, 76: 1099-1114 (1994)).
    It is remarkable that the cAMP signal transduction
5   pathway, including its nuclear components, seem to be
required for memory-related functions in each of these
species and behavioral tasks. Taken together with cellular
analyses of a long-lasting form of LTP in hippocampal
slices (Frey, U., *et al.*, *Science*, 260: 1661-1664 (1993);
10 Huang, Y.Y. and E.R. Kandel, *In Learning and Memory*,
vol. 1, pp. 74-82, Cold Spring Harbor Press, Cold Spring
Harbor, NY (1994)), the emerging picture is that
cAMP-responsive transcription is a conserved molecular
switch involved in the consolidation of short-term memory
15 to long-term memory. Thus, it is reasonable to infer that
differential regulation of CREB isoforms serves as a
molecular switch for the formation of long term memory.
    A universal property of memory formation is that
spaced training (repeated training sessions with a rest
20 interval between them) produces stronger, longer-lasting
memory than massed training (the same number of training
sessions with no rest interval) (Ebbinghaus, H., *Uber das
Gedachtnis*, Dover, New York (1885); Hintzman, D.L., *In
Theories in Cognitive Psychology: The Loyola Symposium*,
25 R.L. Solso (Ed.), pp. 77-99, Lawrence Erlbaum Assoc.,
Hillsdale, New Jersey (1974); Carew, T.J., *et al.*, *Science*,
175: 451-454 (1972); Frost, W.N., *et al.*, *Proc. Natl. Acad.
Sci. USA*, 82: 8266-8269 (1985)). This phenomenon also
exists in fruit flies for a conditioned odor avoidance
30 response (Tully, T. and W.G. Quinn, *J. Comp. Physiol.* 157:
263-277 (1985)). Genetic dissection of this long-lasting
memory has revealed, however, an important difference
between massed and spaced training. Spaced training
produces two functionally independent forms of consolidated

-23-

memory, ARM and LTM, while massed training produces only
ARM.

As described herein, ARM and LTM differ primarily in
their requirement for protein synthesis during induction.
5   ARM is not affected when flies are fed the protein
synthesis inhibitor cycloheximide (CXM) immediately before
or after training, while LTM is completely blocked under
the same feeding conditions. ARM in normal flies also
decays away within four days after training, while LTM
10   shows no decay for at least seven days. Thus, protein
synthesis is required to induce LTM, but LTM is maintained
indefinitely once formed. These latter properties of LTM
have been observed throughout the animal kingdom (Davis,
H.P. and L.R. Squire, *Psychol. Bull.*, *96*: 518-559 (1984);
15   Castellucci, V.F., *et al.*, *J.Neurobiol.*, *20*: 1-9 (1989);
Erber, J., *J.Comp.Physiol.Psychol.*, *90*: 41-46 (1976);
Jaffe, K., *Physiol.Behav.*, *25*: 367-371 (1980)). The
emerging neurobiological interpretation is that formation
of LTM involves protein synthesis-dependent structural
20   changes at relevant synapses (Greenough, W.T., *TINS, 7*:
229-283 (1984); Buonomano, D.V. and J.H. Byrne, *Science*,
249: 420-423 (1990); Nazif, F.A., *et al.*, *Brain Res.*, *539*:
324-327 (1991); Stewart, M.G., *In Neural and Behavioural
Plasticity: The Use of the Domestic Chick As A Model*, R.J.
25   Andrew (Ed.), pp. 305-328, Oxford, Oxford (1991); Bailey,
C.H. and E.R. Kandel, *Sem.Neurosci.*, *6*:35-44 (1994)). The
modern molecular view is that regulation of gene expression
underlies this protein synthesis-dependent effect (Goelet,
P. *et al.*, *Nature, 322*: 419-422 (1986); Gall, C.M. and J.C.
30   Lauterborn, *In Memory:Organization and Locus of Change*,
L.R. Squire, *et al.*, (Eds.) pp.301-329 (1991); Armstrong,
R.C. and M.R. Montminy, *Annu.Rev.Neurosci.*, *16*: 17-29
(1993)).

Why is spaced training required to induce LTM? The
35   massed and spaced procedures both entail ten training

-24-

sessions; consequently, flies receive equivalent exposure
to the relevant stimuli (one odor temporally paired with
electric shock and a second odor presented without shock).
The only procedural difference between massed and spaced
5   training is the rest interval between each training
session. The absence of a rest interval between sessions
during massed training does not appear to disrupt the
memory formation process. The level of initial learning
assayed immediately after massed training is similar to
10 that after spaced training. In addition, ARM levels are
similar after both training procedures. Thus, the presence
of a rest interval during spaced training seems crucial to
the induction of LTM.

        To investigate the temporal kinetics of this rest
15 interval in relation to the formation of LTM (Figures 13A
and 13B), it was first established that the usual ten
sessions of spaced training produced maximal 7-day memory
retention (7-day retention is composed solely of LTM, since
ARM decays to zero within four days.

20   Figure 13A shows that 15 or 20 training sessions did
not improve memory retention. Thus, ten spaced training
sessions produces maximal, asymptotic levels of LTM.

        LTM as a function of the length of the rest interval
between 10 spaced training sessions was then assessed.
25 Figure 13B reveals a continuous increase in LTM from a 0-
min rest interval (massed training) to a 10-minute rest
interval, at which time LTM levels reach maximum. Longer
rest intervals yielded similar memory scores. These
observations of LTM formation suggest an underlying
30 biological process, which changes quantitatively during the
rest interval between sessions and which accumulates over
repeated training sessions.

        In transgenic flies, the formation of LTM, but not ARM
or any other aspect of learning or memory, is disrupted by
35 induced expression of a repressor form of the cAMP-

-25-

responsive transcription factor CREB (Example 4). Mutating
two amino acids in the "leucine zipper" dimerization domain
of this CREB repressor was sufficient to prevent the
dominant-negative effect on LTM. Thus, indication of LTM
5   is not only protein synthesis-dependent but also is CREB-
dependent. Stated more generally, CREB function is
involved specifically in a form of a memory that is induced
only by spaced training. This observation was particularly
intriguing in light of the molecular nature of CREB.
10   In *Drosophila*, transcriptional and/or post-
translational regulation of *dCREB2* yields several mRNA
isoforms. Transient transfection assays in mammalian F9
cells have demonstrated that one of these isoforms (CREB2-
a) functions as a cAMP-responsive activator of transcrip-
15   tion, while a second isoform (CREB2-b) acts as an
antagonistic repressor of the activator (Example 1; cf.
Habener, J.F., *Mol. Endocrinol.*, 4: 1087-1094 (1990);
Foulkes, N. and P. Sassone-Corsi, *Cell, 68*: 411-414
(1992)). (This repressor isoform was used previously to
20   generate the inducible transgene mentioned above.) The
existence of different CREB isoforms with opposing
functions suggested an explanation for the requirement of
multiple training sessions with a rest interval between
them for the formation of LTM.
25   In its simplest form, this model (Example 7; Figure
14) supposes that cAMP-dependent protein kinase (PKA),
activated during training, induces the synthesis and/or
function of both CREB activator and repressor isoforms (cf.
Yamamoto, K.K., *et al.*, *Nature, 334*: 494-498 (1988);
30   Backsai, B.J. *et al.*, *Science, 260*: 222-226 (1993)).
Immediately after training, enough CREB repressor exists to
block the ability of CREB activator to induce downstream
events. Then, CREB repressor isoforms are inactivated
faster than CREB activator isoforms. In this manner, the
35   net amount of functional activator ($\Delta C = CREB2a - CREB2b$)

-26-

increases during a rest interval and then accumulates over
repeated training sessions (with a rest interval) to induce
further the downstream targets involved with the formation
of LTM (Montarolo, P.G., et al., *Science*, 234: 1249-1254
5   (1986); Kaang, B.K., et al., *Neuron*, 10: 427-435 (1993)).

This model leads to three predictions, which have been
confirmed. First, if the functional difference between
CREB activator and repressor isoforms is zero ($\Delta C=0$)
immediately after one training session, then additional
10 massed training sessions should never yield LTM. Figure
15A shows that 48 massed training sessions, rather than the
usual 10, still does not produce any 7-day memory
retention. Second, if the amount of CREB repressor is
increased experimentally, ΔC will be negative immediately
15 after training ($\Delta C < 0$). Then, enough CREB repressor may
not decay during a rest interval to free enough CREB
activator for induction of LTM. This has been shown to be
the case for spaced training (15-min rest interval) after
inducing the expression of a *hsp-dCREB2-b* (repressor)
20 transgene three hours before training (Example 4). Third,
if the amount of CREB activator is increased
experimentally, ΔC will be positive immediately after
training ($\Delta C > 0$). This effect, then, should eliminate or
reduce the rest interval required to induce LTM. Figure
25 15B shows the results from recent experiments in which the
expression of a *hsp-dCREB2-a* (activator) transgene was
induced three hours before training. In these transgenic
flies, massed training produced maximal LTM. This effect
appeared not to arise trivially, since olfactory acuity,
30 shock reactivity (Figure 15C) and initial learning were
normal in transgenic flies after heat shock-induction.
Thus, the requirement for a rest interval between training
sessions to induce LTM specifically was eliminated.

Figure 15B also shows that maximal LTM occurred in
35 induced *hsp-dCREB2-a* transgenic flies after just one

training session. The usual requirement for additional training to form a strong, long-lasting memory was no longer necessary. Thus, induced overexpression of a CREB activator has produced in otherwise normal flies, the
5 functional equivalent of a "photographic" memory. This result indicates that the amount of CREB activator present during training -- rather than the amount of activated PKA that reaches CREB in the nucleus, for instance (cf. Backsai, B.J. et al., Science, 260: 222-226 (1993); Kaang,
10 B.K., et al., Neuron, 10: 427-435 (1993); Frank, D.A. and M.E. Greenberg, Cell, 79: 5-8 (1994)) -- is the rate-limiting step of LTM formation. Taken together, results from these experiments support the notion that the opposing functions of CREB activators and repressors act as a
15 "molecular switch" (cf. Foulkes, N.S. et al., Nature, 355: 80-84 (1992)) to determine the parameters of extended training (number of training sessions and rest interval between them) required to form maximum LTM.

To date, seven different dCREB2 RNA isoforms have been
20 identified, and more are hypothesized to exist. Each may be regulated differentially at transcriptional (Meyer, T.E., et al., Endocrinology, 132: 770-780 (1993)) and/or translation levels before or during LTM formation. In addition, different combinations of CREB isoforms may exist
25 in different (neuronal) cell types. Consequently, many different combinations of activator and repressor molecules are possible. From this perspective, the notions that all activators and repressors are induced during a training session or that all repressors inactivate faster than
30 activators (see above) need not be true. Instead, the model requires only that ΔC (the net function of activators and repressors) is less than or equal to zero immediately after training and the increases with time (rest interval).

Theoretically, particular combinations of activator
35 and repressor molecules in the relevant neuron(s) should

-28-

determine the rest interval and/or number of training
sessions necessary to produce maximum LTM for any
particular task or species. Thus, the molecular
identification and biochemical characterization of each
5   CREB activator and repressor isoform used during LTM
formation in fruit flies is the next major step toward
establishing the validity of our proposed model. Similar
experiments in other species may establish its generality.

CREB certainly is not involved exclusively with LTM.
10 The *dCREB2* gene, for instance, is expressed in all fruit
fly cells and probably acts to regulate several cellular
events (Foulkes, N.S. *et al.*, *Nature*, *355*: 80-84 (1992)).

So, what defines the specificity of its effects on
LTM? Specificity most likely resides with the neuronal
15 circuitry involved with a particular learning task. For
olfactory learning in fruit flies, for instance, CREB
probably is modulated via the cAMP second messenger
pathway. Genetic disruptions of other components of this
pathway are known to affect olfactory learning and memory
20 (Livingstone, M.S., *et al.*, *Cell*, *37*: 205-215 (1984);
Drain, P. *et al.*, *Neuron*, *6*: 71-82 (1991); Levin, L.R., *et
al.*, *Cell*, *68*: 479-489 (1992); Skoulakis, E.M., *et al.*,
Neuron 11: 197-208 (1993); Qiu, Y. and R.L. Davis, *Genes
Develop. 7*: 1447-1458 (1993)). Presumably, the stimuli
25 used during conditioning (training) stimulate the
underlying neuronal circuits. The cAMP pathway is
activated in (some) neurons participating in the circuit,
and CREB-dependent regulation of gene expression ensues in
the "memory cells". This neurobiological perspective
30 potentially will be established in *Drosophila* by
identifying the neurons in which LTM-specific CREB function
resides. Experiments using a neuronal co-culture system in
Aplysia already have contributed significantly to this
issue (Alberini, C.M. *et al.*, *Cell*, *76*: 1099-1114 (1994)
35 and references therein).

-29-

The involvement of CREB in memory, or in the structural changes of neurons which underlie memory *in vivo*, also has been implicated in mollusks (Dash, P.K., *Nature*, *345*: 718-721 (1990); Alberini, C.M. et al., *Cell*,
5   *76*: 1099-1114 (1994)) and in mice (Bourtchuladze, R., et al., *Cell, 79*: 59-68 (1994)). Ample evidence also exists for the involvement of the cAMP second messenger pathway in associative learning in *Aplysia* (Kandel, E.R., et al., *In Synaptic Function*, Edelmann, G.M., et al. (Eds.), John
10 Wiley and Sons, New York (1987); Byrne, J.H., et al., *In Advances in Second Messenger and Phosphoprotein Research*, Shenolikar, S. and A.C. Nairn (Eds.), Raven Press, New York, pp. 47-107 (1993)) and in rat hippocampal long-term potentiation (LTP), a cellular model of associative
15 learning in vertebrates (Frey, U., et al., *Science, 260*: 1661-1664 (1993); Huang, Y.Y. and E.R. Kandel, *In Learning and Memory*, vol. 1, pp.74-82, Cold Spring Harbor Press, Cold Spring Harbor, NY (1994)). Finally, cellular and biochemical experiments have suggested that CREB function
20 may be modulated by other second messenger pathways (Dash, P.K., et al., *Proc. Natl. Acad. Sci. USA 88*: 5061-5065 (1991); Ginty, D.D. et al., *Science, 260*: 238-241 (1993); deGroot, R.P. and P. Sassone-Corsi, *Mol. Endocrinol.*, 7: 145-153 (1993)). These observation suggest that CREB might
25 act as a molecular switch for LTM in many species and tasks.

Finally, why might the formation of LTM require a molecular switch? Many associative events occur only once in an animal's lifetime. Forming long-term memories of such
30 events would be unnecessary and if not counterproductive. Instead, discrete events experienced repeatedly are worth remembering. In essence, a recurring event comprises a relevant signal above the noise of one-time events. Teleologically, then, the molecular switch may act as an
35 information filter to ensure that only discrete but

-30-

recurring events are remembered. Such a mechanism would
serve efficiently to tailor an individual's behavioral
repertoire to its unique environment.

The present invention also relates to isolated DNA
5 having sequences which encode (1) a cyclic 3',5'-adenosine
monophosphate (cAMP) responsive transcriptional activator,
or a functional fragment thereof, or (2) an antagonist of a
cAMP responsive transcriptional activator, or a functional
fragment thereof, or (3) both an activator and an
10 antagonist, or functional fragments thereof of both.

The invention relates to isolated DNA having sequences
which encode *Drosophila* dCREB2 isoforms, or functional
analogues of a dCREB2 isoform. As referred to herein, a
functional analogue of a dCREB2 isoform comprises at least
15 one function characteristic of a *Drosophila* dCREB2 isoform,
such as a cAMP-responsive transcriptional activator
function and/or an antagonistic repressor of the cAMP
activator function. These functions (i.e., the capacity to
mediate PKA-responsive transcription) may be detected by
20 standard assays (e.g., assays which monitor for CREB-
dependent activation). For example, assays in F9 cells
have been used extensively to study CREB-dependent
activation because their endogenous cAMP-responsive system
is inactive; (Gonzalez, G.A. *et al.*, *Nature*, 337: 749-752
25 (1989); Masson, N. *et al.*, *Mol. Cell Biol.*, 12: 1096-1106
(1992); Masson, N. *et al.*, *Nucleic Acids Res.*, 21: 1163-
1169 (1993)).

The invention further relates to isolated DNA having
sequences which encode a *Drosophila* dCREB2 gene or a
30 functional fragment thereof. Isolated DNA meeting these
criteria comprise nucleic acids having sequences identical
to sequences of naturally occurring *Drosophila* dCREB2 and
portions thereof, or variants of the naturally occurring
sequences. Such variants include mutants differing by the

-31-

addition, deletion or substitution of one or more nucleic
acids.

The invention relates to isolated DNA that are
characterized by (1) their ability to hybridize to a
5   nucleic acid having the DNA sequence in Figure 1A (SEQ ID
NO.: 1) or its complement, or (2) by their ability to
encode a polypeptide of the amino acid sequence in Figure
1A (SEQ ID NO.: 2) or functional equivalents thereof (i.e.,
a polypeptide which functions as a cAMP responsive
10   transcriptional activator), or (3) by both characteristics.
Isolated nucleic acids meeting these criteria comprise
nucleic acids having sequences homologous to sequences of
mammalian CREB, CREM and ATF-1 gene products. Isolated
nucleic acids meeting these criteria also comprise nucleic
15   acids having sequences identical to sequences of naturally
occurring dCREB2 or portions thereof, or variants of the
naturally occurring sequences. Such variants include
mutants differing by the addition, deletion or substitution
of one or more residues, modified nucleic acids in which
20   one or more residues is modified (e.g., DNA or RNA
analogs), and mutants comprising one or more modified
residues.

Such nucleic acids can be detected and isolated under
high stringency conditions or moderate stringency
25   conditions, for example. "High stringency conditions" and
"moderate stringency conditions" for nucleic acid
hybridizations are explained on pages 2.10.1-2.10.16 (see
particularly 2.10.8-11) and pages 6.3.1-6 in *Current
Protocols in Molecular Biology* (Ausubel, F.M. et al., eds,
30   Vol. 1, Suppl. 26, 1991), the teachings of which are
incorporated herein by reference. Factors such as probe
length, base composition, percent mismatch between the
hybridizing sequences, temperature and ionic strength
influence the stability of nucleic acid hybrids. Thus,
35   high or moderate stringency conditions can be deterimined

-32-

empirically, depending in part upon the characteristics of
the known DNA to which other unknown nucleic acids are
being compared for homology.

     Isolated nucleic acids that are characterized by their
5   ability to hybridize to a nucleic acid having the sequence
in Figure 1A or its complement (e.g., under high or
moderate stringency conditions) may further encode a
protein or polypeptide which functions as a cAMP responsive
transcriptional activator.

10   The present invention also relates to isolated DNA
having sequences which encode an enhancer-specific
activator, or a functional fragment thereof.

    The invention further relates to isolated DNA having
sequences which encode a *Drosophila* dCREB1 gene or a
15   functional fragment thereof. Isolated DNA meeting these
criteria comprise nucleic acids having sequences identical
to sequences of naturally occurring *Drosophila* dCREB1 and
portions thereof, or variants of the naturally occurring
sequences. Such variants include mutants differing by the
20   addition, deletion or substitution of one or more nucleic
acids.

    The invention further relates to isolated DNA that are
characterized by (1) their ability to hybridize to a
nucleic acid having the DNA sequence in Figure 5 (SEQ ID
25   NO.: 7) or its complement, or (2) by their ability to
encode a polypeptide of the amino acid sequence in Figure 5
(SEQ ID NO.: 8), or by both characteristics. Isolated DNA
meeting these criteria also comprise nucleic acids having
sequences identical to sequences of naturally occurring
30   dCREB1 or portions thereof, or variants of the naturally
occurring sequences. Such variants include mutants
differing by the addition, deletion or substitution of one
or more residues, modified nucleic acids in which one or
more residues is modified (e.g., DNA or RNA analogs), and
35   mutants comprising one or more modified residues.

-33-

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions as described above, for example.

Fragments of the isolated DNA which code for
5 polypeptides having a certain function can be identified and isolated by, for example, the method of Jasin, M., et al., U.S. Patent No. 4,952,501.

Nitric Oxide in Invertebrates: Drosophila dNOS Gene Codes
        for a Ca²⁺/Calmodulin-Dependent Nitric Oxide Synthase

10   Nitric oxide (NO) is a gaseous mediator of a wide variety of biological processes in mammalian organisms. Applicants have cloned the Drosophila gene, dNOS, coding for a Ca²⁺/calmodulin-dependent nitric oxide synthase (NOS). Presence of a functional NOS gene in Drosophila
15 provides conclusive evidence that invertebrates synthesize NO and presumably use it as a messenger molecule. Furthermore, conservation of an alternative RNA splicing pattern between dNOS and vertebrate neuronal NOS, suggests broader functional homology in biochemistry and/or
20 regulation of NOS.

        NO is synthesized by nitric oxide synthases (NOSs) during conversion of L-arginine to L-citrulline (Knowels, R.G., et al., Biochem. J., 298: 249 (1994); Nathan, C., et al., J. Biol. Chem., 269: 13725 (1994); Marletta, M.A., J.
25 Biol. Chem., 268: 12231 (1993)). Biochemical characterization of NOSs has distinguished two general classes: (i) constitutive, dependent on exogenous Ca²⁺ and calmodulin and (ii) inducible, independent of exogenous Ca²⁺ and calmodulin. Analyses of cDNA clones have
30 identified at least three distinct NOS genes in mammals (Bredt, D.S., et al., Nature, 351: 714-718 (1991); Lamas, S., et al., Proc. Natl.Acad.Sci. USA, 89: 6348-6352 (1992); Lyons, C.R., et al., J. Biol. Chem., 267: 6370 (1992); Lowenstein, C.J., et al., Proc. Natl. Acad. Sci. USA, 89:

-34-

6711 (1992); Sessa, W.C., *et al., J.Biol.Chem.,* 267: 15274
(1992); Geller, D.A., *et al., Proc. Natl. Acad. Sci. USA,*
90: 3491 (1993); Xie, Q. *et al., Science,* 256: 225-228
(1992)) neuronal, endothelial and macrophage, the former
5 two of which are constitutive and the latter of which is
inducible. The nomenclature for these different isoforms
used here is historical, as it is clear now that one or
more isoforms can be present in the same tissues (Dinerman,
J.L., *et al., Proc. Natl. Acad. Sci. USA,* 91: 4214-4218
10 (1994)).

As a diffusible free-radical gas, NO is a
multifunctional messenger affecting many aspects of
mammalian physiology [for reviews, see Dawson, T.M., *et
al., Ann. Neurol.* 32: 297 (1992); Nathan, C., *FASEB J.* 6:
15 3051 (1992); Moncada, S., *et al., N. Eng. J. Med.,* 329:
2002-2012 (1993); Michel, T., *et al., Amer. J. Cardiol.* 72:
33C (1993); Schuman, E.M., *et al., Annu. Rev. Neurosci.* 17:
153-183 (1994)]. NO originally was identified as an
endothelium-derived relaxing factor responsible for
20 regulation of vascular tone (Palmer, R.M.J., *Nature* 327:
524 (1987); Palmer, R.M.J., *et al., Nature* 333: 664 (1988);
Ignarro, L.J., *et al., Proc. Natl. Acad. Sci. USA,* 84: 9265
(1987)) and as a factor involved with macrophage-mediated
cytotoxicity (Marletta, M.A., *et al., Biochemistry* 21: 8706
25 (1988); Hibbs, J.B., *et al., Biochem. Biophys. Res. Comm.*
157: 87 (1989); Steuhr, D.J., *et al., J. Exp. Med.,* 169:
1543 (1989)). Since NO has been implicated in several
physiological processes including inhibition of platelet
aggregation, promotion of inflammation, inhibition of
30 lymphocyte proliferation and regulation of microcirculation
in kidney (Radomski, M., *et al., Proc. Natl. Acad. Sci. USA*
87: 5193 (1990); Albina, J.E., *J. Immunol.* 147: 144 (1991);
Katz, R., *Am. J. Physiol.* 261: F360 (1992); Ialenti, A., *et
al., Eur. J. Pharmacol.* 211: 177 (1992)). More recently,
35 NO also has been shown to play a role in cell-cell

-35-

interactions in mammalian central and peripheral nervous systems -- in regulating neurotransmitter release, modulation of NMDA receptor-channel functions, neurotoxicity, nonadrenergic noncholonergic intestinal
5   relaxation (Uemura, Y., et al., Ann. Neurol. 27: 620-625 (1990)) and activity-dependent regulation of neuronal gene expression (Uemura, Y., et al., Ann. Neurol. 27: 620 (1990); Dawson, V.L., et al., Proc. Natl. Acad. Sci. USA 88: 6368 (1991); Lei, S.Z., et al., Neuron 8: 1087 (1992);
10 Prast, H., et al., Eur. J. Pharmacol. 216: 139 (1992); Peunova, N., Nature 364: 450 (1993)). Recent reports of NO function in synaptogenesis and in apoptosis during development of the rat CNS (Bredt, D.S., Neuron 13: 301 (1994); Roskams, A.J., Neuron 13: 289 (1994)) suggest that
15 NO regulates activity-dependent mechanism(s) underlying the organization of fine-structure in the cortex (Edelman, G.M., et al., Proc. Natl. Acad. Sci. USA 89: 11651-11652 (1992)). NO also appears to be involved with long-term potentiation in hippocampus and long-term depression in
20 cerebellum, two forms of synaptic plasticity that may underlie behavioral plasticity (Bohme, G.A., Eur. J. Pharmacol. 199: 379 (1991); Schuman, E.M., Science 254: 1503 (1991); O'Dell, T.J., et al., Proc. Natl. Acad. Sci. USA 88: 11285 (1991); Shibuki, K., Nature 349: 326 (1991);
25 Haley, J.E., et al., Neuron 8: 211 (1992); Zhuo, M., Science 260: 1946 (1993); Zhuo, M., et al., NeuroReport 5: 1033 (1994)). Consistent with these cellular studies, inhibition of NOS activity has been shown to disrupt learning and memory (Chapman, P.F., et al., NeuroReport 3:
30 567 (1992); Holscher, C., Neurosci. Lett. 145: 165 (1992); Bohme, G.A., et al., Proc. Natl. Acad. Sci. USA 90: 9191 (1993); Rickard, N.S., Behav. Neurosci. 108: 640-644 (1994)).

    Many of the above conclusions are based on
35 pharmacological studies using inhibitors of nitric oxide

-36-

synthases or donors of NO. Interpretations of such studies
usually are limited because the drugs interact with more
than one target and they cannot be delivered to specific
sites. A molecular genetic approach can overcome these
5 problems, however, by disrupting a specific gene, the
product of which may be one of the drug's targets.
Recently, such an approach has been attempted in mice via
generation of a knock-out mutation of the neuronal NOS
(nNOS) (Huang, P.L., et al., Cell 75: 1273-1286 (1993)).
10 While nNOS mutants appeared fully viable and fertile, minor
defects in stomach morphology and hippocampal long-term
potentiation were detected (Huang, P.L., et al., Cell 75:
1273-1286 (1993); O'Dell, T.J., et al., Science 265: 542-
546 (1994)). Moreover, some NOS enzymatic activity still
15 was present in certain regions of the brain, suggesting a
role for other NOS genes in the CNS. While yielding some
relevant information about one specific component of NO
function, this nNOS disruption existed throughout
development. Consequently, functional defects of NOS
20 disruption in adults could not be resolved adequately from
structural defects arising during development. Genetic
tools exist in Drosophila, in contrast, to limit
disruptions of gene functions temporally or spatially.
        To identify candidate Drosophila NOS homologs, a
25 fragment of the rat neuronal NOS cDNA (Bredt, D.S., et al.,
Nature 351: 714-718 (1991)) was hybridized at low
stringency to a phage library of the Drosophila genome as
described in Example 11. The rat cDNA fragment encoded the
binding domains of FAD and NADPH (amino acids 979 - 1408 of
30 SEQ ID NO.: 11), which are cofactors required for NOS
activity, and therefore were expected to be conserved in
fruit flies. Several Drosophila genomic clones were
identified with the rat probe and classified into eight
contigs. Sequence analysis of three restriction fragments
35 from these genomic clones revealed one (2.4R) with high

-37-

homology to mammalian NOSs. The deduced amino acid
sequence of the ORF encoded within the 2.4R fragment
indicated 40% identity to the rat neuronal NOS and binding
sites for FAD and NADPH.

5   The 2.4R DNA fragment then was used to probe a
Drosophila adult head cDNA library as described in Example
11, and eight clones were isolated. Restriction analysis
indicated that all contained identical inserts and thus,
defined a predominant transcript expressed by this
10   *Drosophila* gene. One clone (c5.3) was sequenced in both
directions. The 4491 bp cDNA contained one long ORF of
4350 bp. The methionine initiating this ORF was preceded
by ACAAG which is a good match to the translation start
consensus (A/CAAA/C) for *Drosophila* genes (Cavener, D.R.,
15   Nucleic Acids Res 15: 1353-1361 (1987)). Conceptual
translation of this ORF yielded a protein of 1350 amino
acids with a molecular weight of 151,842 Da.

Comparison of the amino acid sequence of this deduced
Drosophila protein (DNOS) (SEQ ID NO.: 9) to sequences of
20   mammalian NOSs revealed that DNOS is 43% identical to
neuronal NOS (SEQ ID NO.: 11), 40% identical to endothelial
NOS (SEQ ID NO.: 10) and 39% identical to macrophage NOS
(SEQ ID NO.: 12). It also revealed similar structural
motifs in DNOS (Figure 16A-16C). The C-terminal half of
25   the DNOS protein contains regions of high homology
corresponding to the presumptive FMN-, FAD- and NADPH-
binding sites. Amino acids thought to be important for
making contacts with FAD and NADPH in mammalian NOSs
(Bredt, D.S., et al., Nature 351: 714-718 (1991); Lamas,
30   S., et al., Proc. Natl.Acad.Sci. USA 89: 6348-6352 (1992);
Lyons, C.R., et al., J. Biol. Chem. 267: 6370 (1992);
Lowenstein, C.J., et al., Proc. Natl. Acad. Sci. USA 89:
6711 (1992); Sessa, W.C., et al., J.Biol.Chem. 267: 15274
(1992); Geller, D.A., et al., Proc. Natl. Acad. Sci. USA
35   90: 3491 (1993); Xie, Q. et al., Science 256: 225-228

-38-

(1992)) are conserved in DNOS. The middle section of DNOS, between residues 215 and 746 of SEQ ID NO.: 9, showed the highest similarity to mammalian NOSs: it is 61% identical to the neuronal isoform and 53% identical to endothelial

5 and macrophage isoforms. Sequences corresponding to the proposed heme- and calmodulin-binding sites in mammalian enzymes are well-conserved in DNOS. The region located between residues 643-671 of SEQ ID NO.: 9 has the characteristics of a calmodulin-binding domain (basic,

10 amphiphilic α-helix) (O'Neil, K.T., et al., Trends Biochem. Sci. 15: 59-64 (1990)). The amino acid sequence between these two sites is very well conserved among all four NOS proteins, suggesting the location of functionally important domains such as the arginine-binding site (Lamas, S., et

15 al., Proc. Natl. Acad. Sci. USA 89: 6348-6352 (1992)), tetrahydrobiopterine cofactor binding site or a dimerization domain. DNOS also has a PKA consensus site (Pearson, R.B., Meth. Enzymol. 200: 62-81 (1991)) (at Ser-287 of SEQ ID NO.: 9) in a position similar to neuronal

20 and endothelial NOSs.

The 214 amino acid N-terminal domain of DNOS shows no obvious homology to its equivalent portion of neuronal NOS or to the much shorter N-terminal domains of endothelial and macrophage NOSs. This region of DNOS contains an

25 almost uninterrupted homopolymeric stretch of 24 glutamine residues. Such glutamine-rich domains, found in many Drosophila and vertebrate proteins, have been implicated in protein-protein interactions regulating the activation of transcription (Franks, R.G., Mech. Dev. 45: 269 (1994);

30 Gerber, H.-P., et al., Science 263: 808 (1994); Regulski, M., et al., EMBO J. 6: 767 (1987)). Thus, this domain of DNOS could be involved with protein-protein interactions necessary for localization and/or regulation of DNOS activity.

-39-

The above sequence comparisons suggest that a *Drosophila* structural homolog of a vertebrate NOS gene was identified. The order of the putative functional domains in the DNOS protein is identical to that of mammalian
5   enzymes (Figure 15B). Structural predictions based on several protein algorithms also indicate that general aspects of DNOS protein secondary structure (hydrophobicity plot, distribution of α-helixes and β-strands) from the putative heme-binding domain to the C-terminus are similar
10   to those of mammalian NOSs. DNOS also does not contain a transmembrane domain, as is the case for vertebrate NOSs. In addition to these general characteristics, several aspects of DNOS structure actually render it most like neuronal NOS: (i) the overall sequence similarity, (ii) the
15   similarity of the putative calmodulin-binding site (55% identical to the neuronal NOS vs. 45% identical to endothelial NOS or vs. 27% identical to macrophage NOS) and (iii) the large N-terminal domain. Neuronal NOS and DNOS also do not contain sites for N-terminal myristoylation,
20   which is the case for endothelial NOS (Lamas, S., et al., Proc. Natl. Acad. Sci. USA 89: 6348-6352 (1992)), nor do they have a deletion in the middle of the protein, which is the case for macrophage NOS (Xie, Q. et al., Science 256: 225-228 (1992)).

25   To establish that Applicants putative DNOS protein had nitric oxide synthase activity, the *dNOS* cDNA was expressed in 293 human embryonic kidney cells as described in Example 12, which have been used routinely in studies of mammalian NOSs (Bredt, D.S., et al., Nature 351: 714-718 (1991)).
30   Protein extracts prepared from *dNOS*-transfected 293 cells as described in Example 12, contained a 150 kD polypeptide, which was recognized by a polyclonal antibody raised against the N-terminal domain of DNOS (Figure 17A, lane 293 + dNOS). This immunoreactive polypeptide was of a size
35   expected for DNOS and was absent from cells transfected

-40-

with just the pCGN vector alone (Figure 17A, lane 293 + vector).

Extracts made from dNOS-transfected 293 cells showed significant NO synthase activity, as measured by the L-arginine to L-citrulline conversion assay as described in Example 12 (0.1276 ± 0.002 pmol/mg/min; Figure 17B, group B). [In a parallel experiment, the specific activity of rat neuronal NOS expressed from the same vector in 293 cells was 3.0 ± 0.02 pmol/mg/min, N=4]. DNOS activity was dependent on exogenous Ca^{2+}/calmodulin and on NADPH, two cofactors necessary for activity of constitutive mammalian NOSs (Iyengar, R., Proc. Natl. Acad. Sci. USA 84: 6369-6373 (1987); Bredt, D.S., Proc. Natl. Acad. Sci. USA 87: 682-685 (1990)). DNOS activity was reduced 90% by the Ca^{2+} chelator EGTA (Figure 17B, group C). Also, 500μM N-(6-aminohexyl)-1-naphthalene-sulfonamide (W5), a calmodulin antagonist which inhibits activity of neuronal NOS (Bredt, D.S., Proc. Natl. Acad. Sci. USA 87: 682-685 (1990)), diminished DNOS activity to 18% (0.0222 ± 0.001 pmol/mg/min, N=2). In the absence of exogenous NADPH, DNOS (or nNOS) activity was reduced 20% (0.1061 ± 0.011 pmol/mg/min, N=4 for DNOS; 2.7935 ± 0.033 pmol/mg/min, N=2 for nNOS). DNOS activity also was blocked by inhibitors of mammalian NOSs (Rees, D.D., Br. J. Pharmacol., 101: 746-752 (1990)). N^{G}-nitro-L-arginine methyl ester (L-NAME) reduced DNOS activity 84% (Figure 17B, group D), and 100 μM N^{G}-monomethyl-L-arginine acetate produced a complete block (0.0001 ± 0.0002 pmol/mg/min, N=2). These enzymatic data demonstrate that DNOS is a Ca^{2+}/calmodulin-dependent nitric oxide synthase.

Northern blot analysis indicated a 5.0 kb dNOS transcript which was expressed predominantly in adult fly heads but not bodies (Figure 18A). More sensitive RT-PCR experiments as described in Example 13, however, detected dNOS message in poly(A)· RNA from fly bodies. Neuronal NOS

-41-

genes from mice and humans produce two alternatively spliced transcripts, the shorter one of which yields a protein containing a 105 amino acid in-frame deletion (residues 504-608 in mouse or rat neuronal NOS) (Ogura, T.,

5   Biochem. Biophys. Res. Commun. 193: 1014-1022 (1993)). RT-PCR amplification of *Drosophila* head mRNA produced two DNA fragments: the 444 bp fragment corresponded to vertebrate long form and the 129 bp fragment corresponded to vertebrate short form (Figure 18B). Conceptual translation

10   of the 129 bp sequence confirmed a splicing pattern identical to that for the nNOS gene (Figure 18C). Presence of the short NOS isoform in *Drosophila* strengthens the notion that it may play an important role in NOS biochemistry.

15   The discovery of a NOS homolog in *Drosophila* provides definitive proof that invertebrates produce NO and, as suggested by recent reports, most likely use it for intercellular signaling. These data also suggest that a NOS gene was present in an ancestor common to vertebrates

20   and arthropods, implying that NOS has existed for at least 600 million years. Thus, it is expected that NOS genes are prevalent throughout the animal kingdom.

       Consistent with this view are existing histochemical data. NOS activity has been detected in several

25   invertebrate tissue extracts: in *Lymulus polyphemus* Radomski, M.W., Philos. Trans. R. Soc. Lond. B. Biol. Sci., 334: 129-133 (1992)), in the locust brain (Elphick, M.R., et al., Brain Res. 619: 344-346 (1993)), in the salivary gland of *Rhodnius prolixus* (Ribeiro, J.M.C., et al., FEBS

30   Let. 330: 165-168 (1993)(34)) and in various tissues of *Lymnaea stagnalis* (Elofsson, R., et al., NeuroReport 4: 279-282 (1993)). Applications of NOS inhibitors or NO-generating substances have been shown to modulate the activity of buccal motoneurones in *Lymnaea stagnalis*

35   (Elofsson, R., et al., NeuroReport 4: 279-282 (1993)) and

-42-

the oscillatory dynamics of olfactory neurons in
procerebral lobe of *Limax maximus* (Gelperin, A., Nature
369: 61-63 (1994)). NADPH-diaphorase staining, a
relatively specific indicator of NOS protein in fixed
5   vertebrate tissue samples (Dawson, T.M., et al., Proc.
Natl. Acad. Sci. USA 88: 7797 (1991); Hope, B.T., et al.,
Proc. Natl. Acad. Sci. USA 88: 2811 (1991)), also has
suggested the presence of NOS in *Drosophila* heads (Muller,
U., Naturwissenschaft 80: 524-526 (1993)). The present
10 molecular cloning of *dNOS* considerably strengthens the
validity of these observations.
        Sophisticated genetic analyses of NOS function are
available in *Drosophila*. Classical genetics will allow the
creation of point mutations and deletions in *dNOS*,
15 resulting in full or partial loss of *dNOS* function. Such
mutations will permit detailed studies of the role of NOS
during development.
        The invention further relates to isolated DNA that are
characterized by by their ability to encode a polypeptide
20 of the amino acid sequence in Figure 16A-16C (SEQ ID NO.:
9) or functional equivalents thereof (i.e., a polypeptide
which synthesizes nitric oxide). Isolated DNA meeting this
criteria comprise amino acids having sequences homologous
to sequences of mammalian NOS gene products (i.e.,
25 neuronal, endothelial and macrophage NOSs). The DNA
sequence represented in SEQ ID NO.: 25 is an example of
such an isolated DNA. Isolated DNA meeting these criteria
also comprise amino acids having sequences identical to
sequences of naturally occurring dNOS or portions thereof,
30 or variants of the naturally occurring sequences. Such
variants include mutants differing by the addition,
deletion or substitution of one or more residues, modified
nucleic acids in which one or more residues is modified
(e.g., DNA or RNA analogs), and mutants comprising one or
35 more modified residues.

-43-

Such nucleic acids can be detected and isolated under high stringency conditions or moderate stringency conditions, for example. "High stringency conditions" and "moderate stringency conditions" for nucleic acid
5 hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. et al., eds, Vol. 1, Suppl. 26, 1991), the teachings of which are incorporated herein by reference. Factors such as probe
10 length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, high or moderate stringency conditions can be deterimined empirically, depending in part upon the characteristics of
15 the known DNA to which other unknown nucleic acids are being compared for homology.

Isolated DNA that are characterized by their ability to encode a polypeptide of the amino acid sequence in Figure 16A-16C, encode a protein or polypeptide having at
20 least one function of a *Drosophila* NOS, such as a catalytic activity (e.g., synthesis of nitric oxide) and/or binding function (e.g., putative heme, calmodulin, FMN, FAD and NADPH binding). The catalytic or binding function of a protein or polypeptide encoded by hybridizing nucleic acid
25 may be detected by standard enzymatic assays for activity or binding (e.g., assays which monitor conversion of L-arginine to L-citrulline). Functions characteristic of dNOS may also be assessed by in vivo complementation activity or other suitable methods. Enzymatic assays,
30 complementation tests, or other suitable methods can also be used in procedures for the identification and/or isolation of nucleic acids which encode a polypeptide having the amino acid sequence in Figure 16A-16C or functional equivalents thereof.

-44-

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

5   The following materials and methods were used in the work described in Examples 1 and 2.

Expression Cloning of dCREB1 and dCREB2
        Standard protocols for expression cloning by DNA-binding (Ausubel, F., Current Protocols in Molecular
10 Biology, John Wiley and Sons, New York, 1994; Singh, H. et al., Cell, 52: 415-423 (1988)) were followed except as noted. A double-stranded, 3xCRE oligonucleotide was synthesized and cloned between the XbaI and KpnI sites of pGEM7Zf+ (Promega). The sequence of one strand of the
15 oligonucleotide was 5' CGTCTAGATCTATGACTGAATA TGACGTAATATGACGTAATGGTACCAGATCTGGCC 3' (SEQ ID NO.: 17), with the CRE sites underlined. The oligonucleotide was excised as a BglII/HindIII fragment and labeled by filling-in the overhanging ends with Klenow fragment in the
20 presence of [α^{32}P]dGTP, [α^{32}P]dCTP and unlabeled dATP and dTTP (Ausubel, F., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1994)). Just prior to use, the labeled fragment was pre-absorbed to blank nitrocellulose filters to reduce background binding. All
25 other steps were as described (Ausubel, F., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1994)). After secondary and tertiary lifts, positive clones were subcloned into pKS+ (Stratagene) and sequences.

Gel Shift Analysis
30   Gel-mobility shift assays were performed as in Ausubel, F., Current Protocols in Molecular Biology, John

-45-

Wiley and Sons, New York, 1994, with the following
modifications. The 4% polyacrylamide gel (crosslinking
ratio 80:1) was cast and run using 5x Tris-glycine buffer
(Ausubel, F., Current Protocols in Molecular Biology, John
5 Wiley and Sons, New York, 1994)) supplemented with 3mM
MgCl$_2$. The oligonucleotides used as the DNA probes were
boiled and slowly cooled to room temperature at a
concentration of 50 μg/ml in 0.1M NaCl. 50 ng of double-
stranded probe was end-labeled using polynucleotide kinase
10 in the presence of 100 μCi of [γ^{32}P]ATP. The double-
stranded oligonucleotides were purified on a native
polyacrylamide gel and used in a mobility shift assay at
about 0.5 ng/reaction.

For dCREB2, the original dCREB2-b cDNA was subcloned
15 and subjected to site-directed mutagenesis to introduce
restriction sites immediately 5' and 3' of the open reading
frame. This open reading frame was subcloned into the
pET11A expression vector (Novagen) and used to induce
expression of the protein in bacteria. The cells
20 containing this vector were grown at 30°C to an approximate
density of 2x10^8/ml and heat-induced at 37°C for 2 hours.
The cells were collected by centrifugation and lysed
according to Buratowski, S. et al., Proc. Natl. Acad. Sci.,
USA, 88: 7509-7513 (1991). The crude extract was clarified
25 by centrifugation and loaded onto a DEAE column previously
equilibrated with 50 mM TrisHCl, pH 8.0, 10% sucrose, 100
mM KCl. Step elutions with increasing amounts of KCl in
the same buffer were used to elute the dCREB2-b protein,
which was assayed using the gel mobility-shift assay. The
30 peak fraction was dialyzed against the loading buffer and
used in the binding experiment. The specific competitor
that was used was the wild-type CRE oligonucleotide. The
sequence of one strand of the double-stranded
oligonucleotides used in the gel shift analysis are listed.

-46-

For the first two oligonucleotides, wild-type and mutant
CREs are underlined.

Wild-type 3xCRE (SEQ ID NO.: 18):

5'AAA<u>TGACGTAA</u>CGGAAA<u>TGACGTAA</u>CGGAAA<u>TGACGTAA</u>CG 3';

5   Mutant 3xmCRE (SEQ ID NO.: 19):

5' AAA<u>TGAATTAA</u>CGGAAA<u>TGAATTAA</u>CGGAAA<u>TGAATTAA</u>CGG 3';

Nonspecific competitor #1 (SEQ ID NO.: 20):

5' TGCACGGGTTTTCGACGTTCACTGGTAGTGTCTGATGAGGCCGAAAGGCCGAAA

CGCGATCGCCATAACCACCACGCTCAG 3';

10   Nonspecific competitor #2 (SEQ ID NO.: 21):

5'TCGACCCACAGTTTCGGGTTTTCGAGCAAGTCTGCTAGTGTCTGATGAGGCCG

AAAGGCCGAAACGCGAAGCCGTATTGCACCACGCTCATCGAGAAGGC 3';

Nonspecific competitor #3 (SEQ ID NO.: 22):

5' CTAGAGCTTGCAAGCATGCTTGCAAGCAAGCATGCTTGCAAGCATGCTTG

15 CAAGC 3';

Nonspecific competitor #4 (SEQ ID NO.: 23):

5' CTCTAGAGCGTACGCAAGCGTACGCAAGCGTACG 3'

For *dCREB1*, heat-induced bacterial extracts (Ausubel,
F., <u>Current Protocols in Molecular Biology</u>, John Wiley and
20 Sons, New York, 1994) were made from the original phage
clone integrated by lysogeny. Extract from a bacteria
lysogenized with another plaque (which did not bind to CRE
sites) from the screen was used as a negative control.
Competition experiments were done using a 4-100 fold molar
25 excess (relative to the probe) of unlabeled, wild-type CRE
oligonucleotides or unlabeled, mutant CRE oligonucleotides.

<u>Northern Blots</u>

Total head and body RNA was isolated from flies
according to the protocol of Drain, P. <u>et al</u>., <u>Neuron</u>,
30 <u>6</u>:71-82 (1991). Total RNA from all other developmental
stages was a gift from Eric Schaeffer. All RNA samples
were selected twice on oligo-dT columns (5 Prime-3 Prime)
to isolate poly A+ RNA. Two μg of poly A+ RNA was
fractionated on 1.2% formaldehyde-formamide agarose gels,

-47-

transferred to nitrocellulose and probed using an uniformly
labeled, strand-specific, antisense RNA (aRNA) probe. The
template for the synthesis of aRNA was one of the partial
cDNA clones isolated from the library screen (pJY199).
5 This cDNA contained the carboxyl-terminal 86 amino acids of
the dCREB2-b protein and about 585 bp of 3' untranslated
mRNA. All Northern blots were washed at high stringency
(0.1% SDS, 0.1xSSC, 65°C).

In situ Hybridization To Tissue Sections
10   Frozen frontal sections were cut and processed under
RNAse-free conditions, essentially as described in Nighorn,
A. et al., _Neuron_, 6:455-467 (1991), with modifications for
riboprobes as noted here. Digoxigenin-labeled riboprobes
were made from pJY199 using the Genius kit (Boehringer-
15 Mannheim). One µg of Xba-linearized template and T3 RNA
polymerase was used to make the antisense probe, while one
µg of EcoRI-linearized template together with T7 RNA
polymerase was used for the control sense probe. Alkaline
hydrolysis (30 minutes at 60°C) was used to reduce the
20 average probe size to about 200 bases. The hydrolyzed
probe was diluted 1:250 in hybridization solution (Nighorn,
A. et al., _Neuron_, 6:455-467 (1991)), boiled, quickly
cooled on ice, added to the slides and hybridized at 42°C
overnight. The slides were then treated with RNAse A (20
25 µg/ml RNAse A in 0.5 M NaCl/10 mM Tris pH8 for 1 hour at
37°C) prior to two 50°C washes. Digoxigenin detection was
as described.

Reverse Transcription Coupled With the Polymerase Chain
Reaction (RT-PCR) Analysis of dCREB2 and Identification of
30 _Alternatively Spliced Exons_
     The template for reverse transcription coupled with
the polymerase chain reaction (RT-PCR) was total RNA or
poly A+ RNA isolated from _Drosophila_ heads as in Drain, P.

-48-

_et al_., _Neuron_, 6: 71-82 (1991). Total RNA used was
exhaustively digested with RNase-free DNase I (50 μg of RNA
digested with 50 units of DNase I for 60-90' at 37°C
followed by phenol, phenol/chloroform extraction, and
5 ethanol precipitation) prior to use. Results from separate
experiments indicate that this DNase-treatment effectively
eliminates the possibility of PCR products derived from any
contaminating genomic DNA. Two rounds of selection using
commercial oligo-dT columns (5 Prime-3 Prime) were used to
10 isolate poly A+ RNA from total RNA. The template for an
individual reaction was either 100-200 ng of total RNA, or
10-20 ng of poly A+ RNA.

The RT-PCR reactions were performed following the
specifications of the supplier (Perkin-Elmer) with a "Hot
15 Start" modification (Perkin-Elmer RT-PCR kit instructions).
All components of the RT reaction, except the rTth enzyme,
were assembled at 75°C, and the reaction was initiated by
adding the enzyme and lowering the temperature to 70°C. At
the end of 15 minutes, the preheated (to 75°C) PCR
20 components (including trace amounts of $[\alpha^{32}P]dCTP$) were
added quickly, the reaction tubes were put into a pre-
heated thermocycler, and the PCR amplification begun.
Cycling parameters for reactions (100 μl total volume) in a
Perkin-Elmer 480 thermocycler were 94°C for 60 seconds,
25 followed by 70°C for 90 seconds. For reactions (50μl) in
an MJ Minicycler the parameters were 94°C for 45 seconds
and 70°C for 90 seconds.

All primers used in these procedures were designed to
have 26 nucleotides complementary to their target sequence.
30 Some primers had additional nucleotides for restriction
sites at their 5' ends to facilitate subsequent cloning of
the products. Primers were designed to have about 50% GC
content, with a G or C nucleotide at their 3' most end and
with no G/C runs longer than 3. For RT-PCR reactions with
35 a given pair of primers, the Mg^{+2} concentration was

-49-

optimized by running a series of pilot reactions, at Mg^{+2}
concentrations ranging from 0.6 mM to 3.0 mM. Reaction
products were analyzed on denaturing urea-polyacrylamide
gels by autoradiography. Any product that appeared larger
5   than the band predicted from the cDNA sequence was purified
from a preparative native gel, re-amplified using the same
primers, gel-purified, subcloned and sequenced.

    To verify that a given RT-PCR product was truly
derived from RNA, control reactions were run to show that
10   the appearance of the product was eliminated by RNase A
treatment of the template RNA. Also, products generated
from reactions using total RNA as the template were re-
isolated from reactions using twice-selected polyA+RNA as
template.

15   Plasmids

    Expression constructs for transient transfection
experiments in Drosophila were made in the expression
vector pAct5CPPA (Han, K. et al., Cell, 56: 573-583 (1989))
or in pAcQ. pAcQ is a close derivative of pAct5CPPA in
20   which the XbaI site at the 5′ end of the 2.5 kb actin
promoter fragment was destroyed and additional sites were
inserted in the polylinker. pAc-dCREB1 was made by
subcloning a KpnI-SacI fragment containing the complete
dCREB1 open reading frame (from a cDNA subcloned into pKS+)
25   into pAct5CPPA. pAc-PKA was constructed by subcloning an
EcoRV fragment encoding the Drosophila PKA catalytic
subunit (Foster, J.L. et al., J. Biol. Chem., 263: 1676-
1681 (1988)) from a modified pHSREM1 construct (Drain, P.
et al., Neuron, 6: 71-82 (1991)) into pAct5CPPA. To make
30   the 3xCRE-lacZ reporter construct for Drosophila cell
culture, the double-stranded, wild-type 3xCRE
oligonucleotide used in the gel shift analysis was cloned
into the KpnI-XbaI backbone of HZ50PL (Hiromi, Y. and W.J.
Gehring, Cell, 50: 963-974 (1987)), a reporter construct

-50-

made for enhancer testing which has cloning sites in front
of a minimal *hsp70* promoter-*lacZ* fusion gene.

     RSV-dCREB2-a was constructed in a long series of
cloning steps. Essentially, the activator-encoding open
5   reading frame was first reconstructed on the plasmid pKS+
by sequentially adding each of the three exons (exons 2, 4
and 6) into the original cDNA of *dCREB2-b*, which had been
subcloned from phage DNA into pKS+. Site-directed
mutagenesis was used to introduce unique restriction enzyme
10   sites both 5' and 3' of the *dCREB2-b* open reading frame,
and these sites facilitated the subcloning process and
allowed removal of 5' and 3' untranslated sequences. Once
the activator was assembled, the resulting open reading
frame was sequenced to confirm the cloning steps and moved
15   into a modified RSV vector which contained a polylinker
located between the RSV promoter and the SV40
polyadenylation sequences (RSV-0). *RSV-dCREB2-b* was made
by moving the original *dCREB2-b* cDNA (which had been
subcloned into pKS+) into RSV-0.

20   Other constructs used in experiments were: pCaE (pMtC)
(Mellon, P.L. *et al.*, *Proc. Natl. Acad. Sci. USA*, *86*: 4887-
4891 (1989)), which contains the cDNA for mouse PKA
catalytic subunit cloned under the mouse metallothionein 1
promoter; RSV-ßgal (Edlund, T. *et al.*, *Science*, *230*: 912-
25   916 (1985)), which expresses the *lacZ* gene under control of
the Rous sarcoma long terminal repeat promoter (Gorman,
C.M. *et al.*, *Proc. Natl. Acad. Sci. USA*, *79*: 6777-6781
(1982)). RSV-CREB (Gonzalez, G.A. *et al.*, *Nature*, *337*:
749-752 (1989)), is a CREB cDNA fragment containing the 341-
30   amino acid open reading frame under the RSV LTR-promoter in
RSV-SG, and the D(-71) CAT reporter (Montminy, M.R. *et al.*,
Proc. Natl. Acad. Sci. USA, *83*: 6682-6686 (1986)) which is
a fusion of a CRE-containing fragment of the rat
somatostatin promoter and the bacterial CAT coding region.

-51-

F9 Cell Culture and Transfection

Undifferentiated F9 cells were maintained and transfected using the calcium phosphate method as described in Darrow, A.L. et al., "Maintenance and Use of F9 Terato-
5 carcinoma Cells" In Meth. Enzymol., v. 190 (1990), except that chloroquine was added to 100 mM immediately before transfection and precipitates were washed off ten hours after transfection, at which time the dishes received fresh chloroquine-free medium. Amounts of DNA in transfections
10 were made equivalent by adding RSV-0 where required. Cells were harvested 30 hours after transfection. Extracts were made by three cycles of freeze/thawing, with brief vortexing between cycles. Particulate matter was cleared from extracts by ten minutes of centrifugation in the cold.
15 ß-galactosidase assays were performed as described in Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972. CAT assays were performed as described in Sheen, J.Y. and B. Seed, Gene, 67: 271-277 (1988) using aliquots of extract
20 heat-treated at 65°C for ten minutes and centrifuged for ten minutes to remove debris. Results reported are from three experiments run on different days with at least three dishes per condition within each experiment. Error bars represent standard error of the mean, with error
25 propagation taken into account (Grossman, M. and H.W. Norton, J. Hered., 71: 295-297 (1980)).

Drosophila Cell Culture and Transient Transfection

Schneider L2 cells in Schneider's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) or Kc167
30 cells in D-22 medium (Sigma) supplemented with 10% FBS, were transfected by the calcium phosphate method essentially as described in Krasnow, M.A. et al., Cell, 57: 1031-1043 (1989), with the following differences. Kc167 cells were plated at 2×10^6 cells/ml and chloroquine was

-52-

added to a final concentration of 100 mM immediately prior
to transfection. A total of 10 μg of plasmid DNA per dish
was used for L2 transfections and 25 μg per dish for Kc167
transfections. DNA masses in transfections were made

5 equivalent with pGEM7Zf+ where required. Precipitates were
left undisturbed on L2 cells until harvest, but for Kc167
cells the original medium was replaced with fresh,
chloroquine-free medium after twelve hours. Cells were
harvested thirty-six to forty-eight hours after

10 transfection. Extracts were made and enzymatic assays
performed as described above for F9 cells. Results
reported for transfections are averages of at least three
experiments run on different days, with at least duplicate
dishes for each condition within experiments. Error bars

15 represent standard error of the mean, with error
propagation taken into account (Grossman, M. and H.W.
Norton, *J. Hered.*, 71: 295-297 (1980)).

ß-galactosidase (ßgal) and Chloramphenicol Acetyl
Transferase (CAT) Assays

20 ß-galactosidase assays were run and activity
calculated as described in Miller, J.H., *Experiments in*
Molecular Genetics, Cold Spring Harbor Laboratory, Cold
Spring Harbor, NY, 1972. CAT assays were performed
essentially according to Sheen, J.Y. and B. Seed, *Gene*, 67:

25 271-277 (1988)) using supernatants from heat-treated
aliquots of extracts (65°C for 10 minutes and then
centrifuged for 10 minutes). Relative activity was
calculated according to Sheen, J.Y. and B. Seed, *Gene*, 67:
271-277 (1988)).

30 PKA-Responsive Transcriptional Activation by *dCREB2-a*
F9 cells were transiently transfected with 10 μg of
D(-71) CAT plasmid as a CRE-directed reporter. 5 μg of
RSV-ßgal reporter was included in each dish as a

-53-

normalization control for transfection efficiency.
Different groups received 8 μg of dCREB2-a expression
vector and 4 μg of PKA expression vector, separately or in
combination. Results are expressed as CAT/βgal enzyme
5   activity ratios, standardized to values obtained with PKA-
transfected dishes.

Transcriptional Effect of dCREB2-b and a Mutant Variant On
PKA-Responsive Activation by dCREB2-a
        F9 cells were transiently cotransfected with 10 μg of
10   D(-71) CAT along with the indicated combinations of the
following expression constructs: RSV-dCREB2-a (5 μg); pMtC
(2 μg); RSV-dCREB2-b (5 μg); and RSV-mLZ-dCREB2-b, which
expresses a leucine-zipper mutant of dCREB2-b (5μg). The
DNA mass for each dish was made up to 27 μg with RSV-O.
15   Other experimental conditions are as described above under
"PKA-Responsive Transcriptional Activation by dCREB2-a".

Transcriptional Activation of a CRE Reporter Gene by dCREB1
in Drosophila Schneider L2 cell culture
        The cells were transiently transfected with a dCREB1
20   expression construct (1 μg), with or without a construct
which expresses Drosophila PKA. 3xCRE-ßgal reporter (1 μg)
and the normalization Ac-CAT reporter (1 μg) were included
in each dish. Expression vectors not present in particular
dishes were replaced by pACQ.

25   Example 1   Isolation and Characterization of dCREB2
        Two different genes were isolated in a DNA-binding
expression screen of a Drosophila head cDNA library using a
probe containing three CRE sites (3xCRE). Many clones were
obtained for the dCREB2 gene, while only one clone was
30   obtained for dCREB1. The dCREB2 clones had two
alternatively-spliced open reading frames, dCREB2-b and
dCREB2-c (see Figure 2). These differed only in the

-54-

presence or absence of exon 4 and in their 5' and 3'
untranslated regions. The inferred translation product of
dCREB2-b showed very high sequence similarity to the amino
acid sequences of the basic region/leucine zipper (bZIP)
5   domains of mammalian CREB (SEQ ID NO.: 4), CREM (SEQ ID
NO.: 5) and ATF-1 (SEQ ID NO.: 6) (see Figure 1B).
        Chromosomal *in situ* hybridization using a *dCREB2* probe
localized the gene to a diffuse band at 17A2 on the X
chromosome, a region which contains several lethal
10   complementation groups (Eberl, D.F. *et al.*, *Genetics*, *30*:
569-583 (1992)).
        To determine the DNA binding properties of *dCREB2-b*,
the DNA binding activity of *dCREB2-b* was assayed using a
gel mobility-shift assay. Bacterial extracts expressing
15   the *dCREB2-b* protein retarded the migration of a
triplicated CRE probe (3xCRE). The protein had lower, but
detectable, affinity for a mutated 3xCRE oligonucleotide.
Competition experiments using unlabeled competitor
oligonucleotides showed that the binding of *dCREB2-b* to
20   3xCRE was specific with higher affinity for CRE sites than
to nonspecific DNA. Together with the conserved amino acid
sequence, this functional similarity suggested that *dCREB2*
was a CREB family member.
        The expression pattern of *dCREB2* was determined by
25   Northern blot analysis of poly A+RNA from various
developmental stages. A complex pattern with at least 12
different transcript sizes was found. Two bands of
approximately 0.8 and 3.5 kb were common to all of the
stages. The adult head contained transcripts of at least
30   six sizes (0.8, 1.2, 1.6, 1.9, 2.3 and 3.5 kb). *In situ*
hybridization to RNA in *Drosophila* head tissue sections
showed staining in all cells. In the brain, cell bodies
but not neuropil were stained.
        dCREB2 has alternatively-spliced forms. Initial
35   transfection experiments showed that the *dCREB2-c* isoform

-55-

was not a PKA-responsive transcriptional activator. This
information, together with the complex developmental
expression pattern and the known use of alternative
splicing of the CREM gene to generate PKA-responsive
5 activators (Foulkes, N. and P. Sassone-Corsi, *Cell*, *68*:
411-414 (1992); Foulkes, N.S. *et al.*, *Nature*, *355*: 80-84
(1992)) suggested that additional domains might be required
to code for an activator.   —·

Reverse transcription coupled with the polymerase
10 chain reaction (RT-PCR) was used to identify new exons.
Comparison of the genomic DNA sequence with that of cDNAs
indicated the general exon/intron organization and assisted
in the search for additional exons. Sense and antisense
primers spaced across the *dCREB2-b* cDNA were synthesized
15 and used pairwise in RT-PCR reactions primed with
Drosophila head RNA. Reactions with primers in exons 5 and
7 (see Figure 2) generated two products, one with the
predicted size (compared with the cDNA clones) and one that
was larger. The larger fragment was cloned and its
20 sequence suggested the presence of exon 6 (see Figure 1A;
SEQ ID NO.: 1). A primer within exon 6 was synthesized,
end-labeled and used to screen a *Drosophila* head cDNA
library. Two clones were isolated, sequenced and found to
be identical. This splicing isoform, *dCREB2-d*, confirmed
25 the splice junctions and exon sequence inferred from the
RT-PCR products.

Initial attempts to isolate exon 2 proved difficult.
The genomic sequence that separated exons 1 and 3 (see
Figure 2) was examined and one relatively extensive open
30 reading frame (ORF) was identified. Three antisense
primers, only one of which lay inside this ORF, were
synthesized based on the intron sequence. Three sets of
RT-PCR reactions were run in parallel, each using one of
the three antisense primers and a sense primer in exon 1.
35 Only the reaction that used the antisense primer in the ORF

-56-

produced a PCR product. The sequence of this product
matched a continuous stretch of nucleotides from the
genomic sequence, extending 3' from exon 1 past the splice
junction in the dCREB2-b cDNA to the location of the
5   antisense primer in the ORF. This fragment suggested that
exon 1 might be extended in some mRNAs by use of an
alternative 5' splice site located 3' to the site used to
make dCREB2-b. Based on the newly-identified exon
sequences, a sense primer was made. This primer was used
10 with an antisense primer in exon 3 to generate a new
product whose sequence established the location of the new
5' splice site. The sequence added to exon 1 by
alternative 5' splice site selection is denoted exon 2.
The exon 2 sequence also showed that the same 3' splice
15 site was used both for the original cDNA and for exon 2.
To independently verify this alternative splicing pattern,
RT-PCR was carried out with a primer that spanned the 3'
splice junction of exon 2 and a primer in exon 1. The
sequence of the product corroborated the splice junctions
20 of exon 2 shown in Figure 1A (SEQ ID NO.: 1).

    To determine if exons 2 and 6 could be spliced into
the same molecule, an RT-PCR reaction was carried out with
primers in exons 2 and 6. The reaction produced a product
of the size predicted by coordinate splicing of these two
25 exons and the identity of this product was confirmed by
extensive restriction analysis.

    dCREB2 is a Drosophila CREB/ATF gene. Figure 1A shows
the DNA sequence (SEQ ID NO.: 1) and inferred amino acid
sequence (SEQ ID NO.: 2) of dCREB2-a, the longest ORF that
30 can result from the identified alternative splicing events.
The indicated translation start site for this ORF is
probably authentic because: i) stop codons occur upstream
from this ATG in all reading frames in our dCREB2 cDNAs
(sequences not shown) ii) this ATG was selected by computer
35 (Sheen, J.Y. and B. Seed, Gene, 67: 271-277 (1988)) as the

-57-

best ribosome binding site in the DNA sequence that
contains the ORF; and iii) use of the next ATG in the open
reading frame 480 nucleotides downstream would not produce
an inferred product that is a PKA-dependent activator (see
5 below). This information does not exclude the possibility
that internal translation initiation sites may be used in
this transcript, as happens in the CREM gene's S-CREM
isoform (Delmas, V. *et al., Proc. Natl. Acad. Sci. USA, 89:*
4226-4230 (1992)).

10   The *dCREB2-a* open reading frame predicts a protein of
361 amino acids with a carboxyl-terminal bZIP domain (SEQ
ID NO.: 3) highly homologous to those of mammalian CREB
(SEQ ID NO.: 4) and CREM (SEQ ID NO.: 5) (see Figure 1B).
The inferred *dCREB2-a* product has a small region of amino
15 acids containing consensus phosphorylation sites for PKA,
calcium/calmodulin-dependent kinase II (CaM kinase II) and
protein kinase C (PKC) at a position similar to that of the
P-box in CREB, CREM and ATF-1. The amino-terminal third of
the predicted *dCREB2-a* is rich in glutamines (including
20 runs of four and five residues). Glutamine-rich activation
domains occur in CREB, CREM, and other eukaryotic
transcription factors, including some from *Drosophila*
(Courey, A.J. and R. Tijan, "Mechanisms of Transcriptional
Control as Revealed by Studies of the Human Transcription
25 Factor Sp1" *In Transcriptional Regulation,* vol. 2,
McKnight, S.L. and K.R. Yamamoto (eds.), Cold Spring Harbor
Press, Cold Spring Harbor, NY, 1992; Mitchell, P.J. and R.
Tijan, *Science, 245:* 371-378 (1989)).
A computerized amino acid sequence homology search
30 with the predicted *dCREB2-a* protein sequence (SEQ ID
NO.: 2) identifies CREB, CREM and ATF-1 gene products as
the closest matches to *dCREB2-a.* The homology is
particularly striking in the carboxyl-terminal bZIP domain
where 49 of 54 amino acids are identical with their
35 counterparts in mammalian CREB (Figure 1B). The homology

-58-

is less striking, albeit substantial, in the activation domain. Lower conservation in this domain is also characteristic of the mammalian CREB and CREM genes (Masquilier, D. et al., *Cell Growth Differ.*, 4: 931-937
5   (1993)).

Figure 2 shows the exon organization of all of the *dCREB2* alternative splice forms that we have detected, both as cDNAs and by RT-PCR. Splice products of *dCREB2* fall into two broad categories. One class of transcripts
10   (*dCREB2-a*, *-b*, *-c*, *-d*) employs alternative splicing of exons 2, 4 and 6 to produce isoforms whose protein products all have the bZIP domains attached to different versions of the activation domain. The second class of transcripts (*dCREB2-q*, *-r*, *-s*) all have splice sites which result in
15   in-frame stop codons at various positions upstream of the bZIP domain. These all predict truncated activation domains without dimeriation or DNA binding activity.

Two different *dCREB2* isoforms, *dCREB2-a* and *dCREB2-b*, have opposite roles in PKA-responsive transcription. The
20   capacity of isoforms of the *dCREB2* gene to mediate PKA-responsive transcription was tested in F9 cells. These cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive transcription system is inactive (Gonzalez, G.A. et al.,
25   *Nature*, 337: 749-752 (1989); Masson, N. et al., *Mol. Cell Biol.*, 12: 1096-1106 (1992); Masson, N. et al., *Nucleic Acids Res.*, 21: 1163-1169 (1993)). Candidate cAMP-responsive transcription factors, synthesized from expression vectors, were transiently transfected with and
30   without a construct expressing the PKA catalytic subunit. CREB-dependent changes in gene expression were measured using a cotransfected construct that has a CRE-containing promoter fused to a reporter gene.

The product of the *dCREB2-a* isoform is a PKA-dependent
35   activator of transcription (Figure 3). Transfection of PKA

-59-

or *dCREB2-a* alone gave only modest activation above
baseline values. Cotransfection of *dCREB2-a* and PKA
together, however, gave levels of activation 5.4-fold
greater than the activation seen with PKA alone.

5   *dCREB2-b* did not act as a PKA-dependent
transactivator. When transfected together with the
reporter and PKA, it failed to stimulate reporter activity.
Instead, it functioned as a direct antagonist of PKA-
dependent activation by *dCREB2-a* (Figure 4). Cotranfection
10 of equimolar amounts of the *dCREB2-a* and *dCREB2-b*
expression constructs, along with PKA and the reporter,
resulted in a nearly complete block of PKA-dependent
activation from the CRE-containing reporter.

       The strong homology between the leucine zippers of
15 *dCREB2* (SEQ ID NO.: 3), CREB (SEQ ID NO.: 4) and CREM (SEQ
ID NO.: 5) (see Figure 1B) suggested that mutations which
abolish CREB dimerization (Dwarki, V.J. et al., *EMBO J.*, *9*:
225-232 (1990)) should also affect *dCREB2* dimerization.
The mutant *Drosophila* molecule mLZ-*dCREB2-b* was made by
20 introducing two single-base changes that convert the middle
two leucines of the leucine zipper to valines. An
identical mutation in CREB abolishes homodimerization *in
vitro* (Dwarki, V.J. et al., *EMBO J.*, *9*: 225-232 (1990)).
Cotransfected mLZ-*dCREB2-b* failed to block PKA-dependent
25 activation by *dCREB2-a* (Figure 4).

       <u>Example 2   <u>Isolation and Characterization of *dCREB1*</u></u>
       A single cDNA representing the *dCREB1* gene was
isolated in the same screen of a *Drosophila* lambda gt11
expression library that yielded the *dCREB2* cDNAs. The
30 sequence of the *dCREB1* cDNA contained a complete open
reading frame specifying a 266 amino acid protein with a
carboxyl-terminal leucine zipper four repeats long and an
adjacent basic region (Figure 5; SEQ ID NO.: 7). The
amino-terminal half of the inferred protein contains an

-60-

acid-rich activation domain, with glutamate, asparate and proline residues spaced throughout. dCREB1 has consensus phosphorylation sites for CaM kinase II and PKC throughout its length, but has no predicted phosphoacceptor site for
5 PKA.
        Gel shift analysis showed higher-affinity binding of the dCREB1 protein to 3xCRE than to 3xmCRE. Transcriptional activation by dCREB1 was assayed with transient cotransfection experiments using the Drosophila
10 L2 and Kc167 cell culture lines. In L2 cells, dCREB1 activates transcription from CREs, but this effect is not enhanced by cotransfection of PKA (Figure 6). In Kc167 cells, dCREB1 fails to activate reporter expression either alone or with cotransfected PKA expression constructs.
15   Genomic Southern blot analysis indicates that dCREB1 is a single copy gene, and chromosomal in situ hybridization shows that it is located at 54A on the right arm of chromosome 2.
        These results show that dCREB1 is a non-PKA responsive
20 CREB family member from Drosophila.

        The following materials and methods were used in the work described in Examples 3 and 4.

Isolating Transgenic Flies
        EcoRI restriction sites were added (using PCR) just 5'
25 to the putative translation initiation site and just 3' to the translation termination site in the dCREB2-b cDNA. This fragment was sequenced and subcloned into CaSpeR hs43, a mini-white transformation vector which contains the hsp70 promoter, in the orientation so that the dCREB2-b open
30 reading frame is regulated by the hsp70 promoter. Germ-line transformation was accomplished using standard techniques (Spradling, A.C. and G.M. Rubin, Science, 218: 341-347 (1982); Rubin, G.M. and A. Spradling, Science, 218:

-61-

348-353 (1982)). Two transgenic lines, *17-2* and *M11-1*,
each with one independent P-element insertion were
generated and characterized. They appeared normal in
general appearance, fertility and viability. These
5 transgenic lines were outcrossed for at least five
generations to w(CS-10) (Dura, J-M., et al., J. Neurogenet.
9: 1-14 (1993)), which itself had been outcrossed for ten
generations to a wild-type (Can-S) stock. This extensive
series of outcrossing is necessary to equilibrate the
10 genetic background to that of Canton-S. Flies homozygous
for the 17-2 transgene were bred and used for all
experiments.

    The mutant blocker has been described previously (see
Example 1). The mutations were substituted into an
15 otherwise wild-type blocker construct and germ-line
transformants were made by injecting into w(isoCJ1)
embryoes. Flies homozygous for the A2-2 transgene
insertion were bred and used for all experiments.
w(isoCJ1) is a subline of w(CS10) (see above) carrying
20 isogenic X, 2nd and 3rd chromosomes and was constructed by
Dr. C. Jones in our laboratory. Originally 40 such
sublines were w(CS10) using standard chromosome balancer
stocks. Olfactory acuity, shock reactivity, learning and
three-hr memory after one-cycle training then were assayed
25 in each isogenic subline. As expected, a range of scores
among the sublines was obtained. w(isoCJ1) yielded scores
that were most like those of w(CS10) on each of these
assays. By injecting DNA into the relatively homogeneous
genetic background of w(isoCJ1), outcrossing of the
30 resulting germ-line transformants to equilibrate
heterogeneous) genetic backgrounds was not necessary.

Cycloheximide Feeding and Heat-Shock Regimen
    For experiments on memory retention after one-cycle
training and on retrograde amnesia, flies were fed 35 mM

-62-

cycloheximide (+CMX; Sigma) in 4% sucrose (w/v) or 4%
sucrose alone (-CXM) at 25°C. Groups of 100 flies were
placed in feeding tubes (Falcon 2017) containing two 1.0 x
2.5 cm Whatmann 3MM filter paper strips that were soaked
5   with a total of 250 μl of solution.

For experiments on one day retention after massed or
spaced training, flies were fed 35 mM CXM and (w/v) 5%
glucose dissolved in 3% ethanol. Groups of 100 flies were
placed in feeding tubes (Falcon 2017) containing one 1.0 x
10 2.5 cm Whatmann 3MM filter paper strips that was soaked
~~with a total of 126 μl of solution.~~

For experiments on learning after one-cycle training,
olfactory acuity, and shock reactivity, flies were fed a 5%
glucose, 3% ethanol solution alone or 35 mM CXM in the
15 glucose/ethanol solution.

The feeding period was limited to 12-14 hrs prior to
training, or to the 24-hr retention interval after
training. Flies which were fed prior to training were
transferred directly to the training apparatus after
20 feeding, subjected to massed or spaced training, then
transferred to test tubes containing filter paper strips
soaked with 5% glucose during the 24-hr interval. Flies
which were fed after training were trained, then
transferred immediately to test tubes containing filter
25 paper strips soaked with 5% glucose solution which was
laced with 35 mM CXM. Flies remained in the test tubes for
the duration of the 24-hr retention interval.

For heat-shock induction, flies were collected within
two days of eclosion, placed in glass bottles in groups of
30 about 600, and incubated overnight at 25° C and 70%
relative humidity. The next day, three hours before
training, groups of approximately 100 flies were
transferred to foam-stoppered glass shell vials containing
a strip of filter paper to absorb excess moisture. The
35 vials then were submerged in a 37°C water bath until the

-63-

bottom of the foam stopper (inside the vial) was below the surface of the water, thereby insuring that the flies could not escape heat-shock. The vial remained submerged for 30 min, after which the flies were transferred to a standard

5 food vial for a 3-hr recovery period at 25° C and 70% relative humidity. Training began immediately after the recovery period.

Pavlovian Learning and Memory and Testing

Flies were trained with an automated version of the

10 learning procedure of Tully, T. and W.G. Quinn, *J. Comp. Physiol.*, *157*: 263-277 (1985). In brief, flies were trapped in a training chamber, the inside of which was covered with an electrifiable copper grid. Groups of about 100 flies were exposed sequentially to two odors [either

15 octanol (OCT) or methylcyclohexanol (MCH)], which were carried through the training chamber in a current of air, for 60 seconds with 45 seconds rest intervals after each odor presenation. During exposure to the first odor, flies also were subjected to twelve 1.5-second pulses of 60 V DC

20 with a 5-second interpulse interval. After training, flies were transferred to food vials for a particular retention interval. Conditioned odor-avoidance responses then were tested by transferring flies to the choice point of a T-maze, where they were exposed simultaneously to OCT and

25 MCH carried in the distal ends of the T-maze arms and out the choice point on converging currents of air. Flies were allowed to distribute themselves in the T-maze arms for two minutes, after which they were trapped in their respective arms, anesthetized and counted. The "percent correct" then

30 was calculated as the number of flies avoiding the shock-paired odor (they were in the opposite T-maze arm) divided by the total number of flies in both arms. (The number of flies left at the choice point, which usually was less than 5%, were not included in this calculation). Finally, a

-64-

performance index (PI) was calculated by averaging the
percent corrects of two reciprocal groups of flies -- one
where OCT and shock were paired, the other where MCH and
shock were paired -- and then by normalizing the average so
5   that a PI=0 represented a 50:50 distribution in the T-maze
and a PI=100 represented 100% avoidance of the shock-paired
odor. For these studies, three different training
protocols were used: 1. One-cycle training consisted of
the training session just described. 2. Massed training
10 consisted of 10 of these training cycles delivered one
right after the other. 3. Spaced training consisted of 10
training cycles with a 15-min rest interval between each.
One-cycle training was used to assay learning, while massed
and spaced was used to assay consolidated memories.

15 Olfactory Acuity and Shock Reactivity

        Odor avoidance responses to OCT or to MCH at two
different concentrations -- one (10^0) usually used in
conditioning experiments and a 100-fold (10^{-2}) dilution
thereof -- were quantified in various groups of flies in
20 the absence of heat shock and 3 hr or 24 hr after heat
shock with the method of Boynton, S. and T. Tully,
Genetics, 131: 655-672 (1992). Briefly, flies are placed
in a T-maze and given a choice between an odor and air.
The odors are naturally aversive, and flies ususally choose
25 air and avoid the T-maze arm containing the odor. For
shock reactivity, flies are given a choice between an
electrified grid in one T-maze arm, and an unconnected grid
in the other. After the flies have distributed themselves,
they are anesthetized, counted and a PI is calculated.

30 Statistical Analyses of Behavioral Data

        Since each PI is an average of two percentages, the
Central Limit Theorem predicts that they should be
distributed normally (see Sokal, R.R. and F.J. Rohlf,

-65-

Biometry, 2nd Edition, W.H. Freeman and Company, New York
(1981)). This expectation was shown to be true by an
empirical determination with data from Tully, T. and W.G.
Quinn, *J. Comp. Physiol.*, 157: 263-277 (1985) and Tully, T.
5   and D. Gold, *J. Neurogenet.*, 9: 55-71 (1993). Thus,
untransformed (raw) data were analyzed parametrically with
JMP2.1 statistical software (SAS Institute Inc., Cary NC).
Since preliminary experiments preceded all of the
experiments summarized herein, all pairwise comparisons
10   were planned. To maintain an experimentwise error rate of
alpha = 0.05, the critical P values for these individual
comparisons were adjusted accordingly (Sokal, R.R. and F.J.
Rohlf, *Biometry*, 2nd Edition, W.H. Freeman and Company, New
York (1981)) and are listed below for each experiment.
15   All experiments were designed in a balanced fashion
with N=2 PIs per group collected per day; then replicated
days were added to generate final Ns. In each experiment,
the experimenter (M.D.) was blind to genotype.

A.   One-day memory in wild-type flies fed CXM before
20 or immediately after massed or spaced training (Figure 8):
PIs from these four drug treatments (-CXM before, -CXM
after, +CXM before and +CXM after) and two training
procedures (massed and spaced) were subjected to a TWO-WAY
ANOVA with DRUG $(F_{(3,56)} = 8.93; P < 0.001)$ and TRAINing
25 $(F_{(1,56)} = 18.10, P < 0.001)$ as main effects and DRUG x TRAIN
$(F_{(3,56)} = 4.68, P = 0.006)$ as the interaction term. P
values from subsequent planned comparisons are summarized
in Figure 8. The six planned comparisons were judged
significant if $P \leq 0.01$.

30   B.   One-day memory after massed or spaced training in
dCREB2-b transgenic flies (Figures 9A and 9B): In
experiments with the *17-2* transgenic line, PIs from two
strains (Can-S and *17-2*) and four training-regimens

-66-

(spaced-hs, spaced+hs, massed-hs and massed+hs) were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,40)}$ = 1.57; P = 0.22) and TRAINing-regimen ($F_{(3,40)}$ =25.81, P<0.001) as main effects and STRAIN x TRAIN ($F_{(3,40)}$ = 6.62, P = 0.001) as the

5 interaction term. A similar analysis was done with data from the M11-1 transgenic line, yielding STRAIN ($F_{(1,40)}$ = 2.81; P = 0.10), TRAINing-regimen ($F_{(3,40)}$ = 11.97, P < 0.001) and STRAIN x TRAIN ($F_{(3,40)}$ = 3.37, P = 0.03) effects. P values from subsequent planned comparisons are summarized

10 in Figures 9A and 9B. In each experiment, the seven planned comparisons were judged significant if P ≤ 0.01.

   C.   Learning after one-cycle training in 17-2 transgenic flies (Figure 9C): PIs from two strains (Can-S and 17-2) and three heat-shock regimens [-hs, +hs (3 hr)

15 and+hs (24 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,30)}$ = 0.69; P = 0.41) and HEAT-shock regimen ($F_{(2,30)}$ = 10.29, P < 0.001) as main effects and STRAIN x HEAT ($F_{(2,30)}$ = 0.71, P = 0.50) as the interaction term. P values from subsequent planned comparisons are summarized

20 in Figure 9C. The three planned comparisons were judged significant if P ≤ 0.02.

   D.   One-day memory after spaced training in A2-2 transgenic flies (Figure 10): PIs from these three strains [w(isoCJ1), 17-2 and A2-2] and two heat-shock regimens [-hs

25 and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(2,30)}$ = 9.43, P < 0.001) and HEAT-shock regimen ($F_{(1,30)}$ = 9.84, P = 0.004) as main effects and STRAIN x HEAT ($F_{(2,30)}$ = 5.71, P = 0.008) as the interaction term. P values from subsequent comparisons are summarized in Figure

30 10. The six planned comparisons were judged significant if P ≤ 0.01.

-67-

E.   Olfactory acuity in *17-2* flies (Table): PIs from these two strains (Can-S and *17-2*), four different odor-levels (OCT- 10^0, OCT- 10^{-2}, MCH-10^0 and MCH- 10^{-2}) and three heat-shock regimens [-hs. +hs (3 hr) and +hs (24 hr)]
5 were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,184)}$ = 0.12, P = 0.73), ODOR-level ($F_{(3,184)}$ = 126.77, P < 0.001) and HEAT-shock regimen ($F_{(2,184)}$ = 3.55, P = 0.03) as main effects, STRAIN x ODOR ($F_{(3,184)}$ = 1.23, P = 0.30), STRAIN x HEAT ($F_{(2,184)}$ = 0.33, P = 0.72) and ODOR x HEAT ($F_{(6,184)}$ =
10 3.14, P = 0.006) as two-way interaction terms and STRAIN x ODOR x HEAT ($F_{(6,184)}$ = 0.48, P = 0.83) as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The twelve planned comparisons were significant if P ≤ 0.005.

15   F.   Shock reactivity in *17-2* flies (Table): PIs from these two strains (Can-S and *17-2*), two shock groups (60V and 20V) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,84)}$ = 0.50, P = 0.48), SHOCK ($F_{(1,84)}$ = 97.78,
20 P<0.001) and HEAT-shock regimen ($F_{(2,84)}$ = 3.36, P = 0.04) as main effects, STRAIN x SHOCK ($F_{(1,84)}$ = 1.12, P = 0.29), STRAIN x HEAT ($F_{(2,84)}$ = 1.06, P = 0.35) and SHOCK x HEAT ($F_{(2,84)}$ = 6.66, P = 0.002) as two-way interaction terms and STRAIN x SHOCK x HEAT ($F_{(2,84)}$ =1.75, P =0.18) as the
25 three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The six planned comparisons were judged significant if P ≤ 0.01.

G.   Seven-day memory after spaced training in *17-2* flies (Figure 11): PIs from two strains (Can-S and 17-2)
30 and two heat-shock regimens [-hs and +hs(3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,20)}$ = 6.09; P = 0.02) and HEAT-shock regimen ($F_{(1,20)}$ = 16.30, P = 0.001) as main effects and STRAIN x TRAIN ($F_{(1,20)}$ = 7.73, P = 0.01) as

-68-

the interaction term. P values from subsequent planned
comparisons are summarized in Figure 11. The three planned
comparisons were judged significant if P ≤0.02.

H.   One-day memory after spaced training in $rsh;17-2$
5 double mutants (Figure 12): PIs from three strains (17-2,
rsh and $rsh;17-2$) and two heat-shock regimens [-hs and +hs
(3 hr)] were subjected to a TWO WAY ANOVA with STRAIN
($F_{(2,30)}$= 32.05; P < 0.001) and HEAT-shock regimen ($F_{(1,30)}$ =
59.68, P< 0.001) as main effects and STRAIN x TRAIN ($F_{(2,30)}$
10 = 11.59, P < 0.001) as the interaction term. P values from
subsequent planned comparisons are summarized in Figure 12.
The five planned comparisons were judged significant if P ≤
0.01.

I.   Learning after one-cycle training in $rsh;17-2$
15 mutants (see text): PIs from these two strains ($Can-S and$
$rsh;17-2$) and two heat-shock regimens [-hs and +hs (3 hr)]
were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,20)}$ =
86.85, P < 0.001) and HEAT-shock regimen ($F_{(1,20)}$ = 0.02, P <
0.89) as main effects and STRAIN x HEAT ($F_{(1,20)}$ = 0.86, P =
20 0.37) as the interaction term. P values from subsequent
planned comparisons are summarized in the Table. The two
planned comparisons were significant if P ≤ 0.03.

J.   Olfactory acuity in $rsh;17-2$ flies (Table): PIs
from these two strains (Can-S and $rsh;17-2$), four different
25 odor-levels (OCT-10^0, OCT-10^{-2}, MCH-10^0 and MCH-10^{-2}) and two
heat-shock regimens [-hs, and +hs (3 hr)] were subjected to
a THREE-WAY ANOVA with STRAIN ($F_{(1,112)}$ = 0.02, P = 0.88),
ODOR-level ($F_{(3,112)}$ = 50.03, P < 0.001) and HEAT-shock
regimen ($F_{(1,112)}$ = 29.86, P < 0.001) as main effects, STRAIN
30 x ODOR ($F_{3,112}$ = 2.15, P = 0.10), STRAIN x HEAT ($F_{(1,112)}$ =
0.34, P = 0.56) and ODOR x HEAT ($F_{(3,112)}$ = 6.41, P = 0.001)
as two-way interaction terms and STRAIN x ODOR x HEAT

-69-

$(F_{(3,112)} = 1.12, P = 0.35)$ as the three-way interaction term. P values from subsequent planned comparisons are summarized in the Table. The eight planned comparisons were judged significant if $P \leq 0.01$.

5   K.   Shock reactivity in *rsh;17-2* flies (Table): PIs from these two strains (Can-S and *rsh;17-2*), two shock groups (60V and 20V) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to THREE-WAY ANOVA with STRAIN $(F_{(1,56)} = 0.51, P = 0.48)$, SHOCK $(F_{(1,56)} = 88.14, P < 0.001)$
10 and HEAT-shock regimen $(F_{(1,56)} = 0.08, P = 0.77)$ as main effects, STRAIN x SHOCK $(F_{1,56} = 0.12, P = 0.73$, STRAIN x HEAT $(F_{(1,56)} = 0.03, P = 0.86)$ and SHOCK x HEAT $(F_{(1,56)} = 0.39, P = 0.53)$ as two-way interaction terms and STRAIN x SHOCK x HEAT $(F_{(1,84)} = 1.58, P = 0.21)$ as the three-way
15 interaction term. P values from subsequent planned comparisons are summarized in the Table. The four planned comparisons were judged significant if $P \leq 0.01$.

Northern Analysis

      For RNA collection, the heat-shock regimen was the
20 same as for behavioral experiments. For any indicated time interval between heat-shock and collection, flies rested in food-containing vials at 25°C. Flies were collected and quickly frozen in liquid nitrogen. All Northern analyses used head RNA. The tube of frozen flies was repeatedly
25 rapped sharply on a hard surface, causing the heads to fall off. The detached frozen heads were recovered by sieving on dry ice. Approximately 1000 heads were pooled for RNA preparation. Wild-type and transgenic flies for each individual time point always were processed in parallel.
30 Flies that were not induced were handled in a similar manner to induced flies, except that the vials were not placed at 37°C. Total head RNA was isolated from each group of flies, and poly A+ RNA was isolated using oligo dT

-70-

columns according to the instructions of the manufacturer (5'--->3' Inc.). The concentration of poly A+ mRNA was measured spectrophotometrically, and 0.5 mg of mRNA per lane was loaded and run on 1.2% formaldehyde-agarose gels.

5   Northern blots were prepared, probed and washed (0.1 x SSC at 65°C) as described (Ausubel, F., *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 1994). For detection of the transgene, an 843bp dCREB2-b cDNA fragment was subcloned into pKS+ and used to generate a

10 uniformly-labeled antisense riboprobe. This fragment codes for the carboxyl-terminal 86 amino acids of the *dCREB2-b* protein plus 3' untranslated mRNA.

Western Blot Analysis and Antiserum

Western blot analysis was performed using a rabbit

15 antiserum raised against a peptide corresponding to 16 amino acids in the basic region of the *dCREB2-b* cDNA with an additional COOH terminal Cys. The sequence of the peptide was: (SEQ ID NO.: 24) NH2-RKREIRLQKNREAAREC-COOH. The peptide was synthesized and coupled to Sulfo-SMCC

20 (Pierce) activated keyhole lympet hemocyanin. The antigen was injected into rabbits (100 μg) and boosted at two week intervals. Sera was bled and tested for immune reactivity towards bacterially-expressed dCREB2-b protein. The antiserum was passed through a CM Affi-gel Blue column

25 (Biorad), and the flow-through was concentrated by ammonium sulfate precipitation, resuspended and dialyzed against PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, 1.4 mM KH_2PO_4, pH 7.3). The dialyzed serum was affinity-purified using a peptide column made using an Ag/Ab Immobilization kit

30 (Immunopure from Pierce). After the antiserum was eluted using a 4M $MgCl_2$, 0.1 M HEPES pH 6.0 buffer, it was dialyzed into PBS and frozen.

Each data point represents approximately 5 fly heads. Groups of about 25-50 flies were collected and quickly

-71-

frozen on liquid nitrogen until all of the time points had
been collected. Heads were isolated resuspended in
approximately 200 μl of 1x Laemmli sample buffer, allowed
to thaw and homogenized with a Dounce type B pestle.

5   Samples were boiled for 5 minutes, and centrifuged for 10
minutes at room temperature in an Eppendoff
microcentrifuge. The supernatants were collected and
boiled again just prior to loading onto protein gels.
Standard procedures were used to separate equal amounts of

10 proteins from each sample on 12% polyacrylamide-SDS gels
and to transfer them to PVDF membranes by electroblotting
(Ausubel, F., *Current Protocols in Molecular Biology*, John
Wiley and Sons, New York, 1994).

The membranes were blocked for 60 minutes with a 5%

15 BSA solution made up in TBST (10 mM Tris, pH 7.9, 150 mM
NaCl, 0.05% Tween 20). The primary antibody was diluted
1:1000 in TBST and incubated with the filter for 30
minutes. The membranes were washed three times with TBST
for 5 minutes each time and then incubated for 30 minutes

20 with an alkaline phosphatase-conjugated anti-rabbit IgG
second antibody (Promega) diluted 1:7500 in TBST. The
membranes were washed three more times as before and
developed using a chromogenic alkaline phosphatase reaction
according to manufacturers suggestions (Promega).

25 Example 3   Transgene Expression Increased After Heat-
                    Shock Induction
In order to interpret the effects of transgene
induction on behavior, dCREB2-b expression in transgenic
flies *(17-2)* after heat-shock induction was measured.

30 Northern blot analysis revealed elevated levels of hs-
dCREB2-b message in the *17-2* flies immediately and three
hours after heat-shock (Figure 7A). This induction was
also detectable in brain cells using *in situ* hybridization.
Western blot showed increased dCREB2-b protein immediately

-72-

after induction (Figure 7B). Elevated levels of the
dCREB2-b protein were seen nine hours later and were still
detectable twenty four hours after induction. These data
indicate that increased amounts of dCREB2-b existed in
5   brain cells throughout spaced training, which ended about
six hours after heat induction.

The behavioral experiments also used transgenic flies
(A2-2) which expressed a mutated dCREB2-b protein
(dCREB2-mLZ). These mutations changed the two internal
10 leucine residues of the leucine zipper to valine residues,
and these changes have been shown to result in a protein
which is unable to form dimers (Dwarki, V.J. et al., EMBO
J., 9: 225-232 (1990)). In transient co-transfection
assays, the mutant protein was unable to block
15 PKA-dependent transcription mediated by dCREB2-a, while the
wild-type protein had blocking function. Western blot
analysis showed that the wild-type and mutant blocker are
expressed at similar levels beginning immediately after
heat-shock induction and lasting for at least 6 hours
20 (Figure 7C). Therefore, it is unlikely that these two
proteins have large differences in expression levels or
stability in the transgenic flies.

Northern blot analysis of two different housekeeping
genes, myosin light chain (Parker, V.P., et al., Mol. Cell
25 Biol., 5: 3058-3068 (1985)) and elongation factor α
(Hovemann, B., et al., Nucleic Acids Res., 16: 3175-3194
(1988)), showed that steady-state levels of their RNAs were
unaffected after transgene induction for at least 3 hours.
Gel shift analysis using two different consensus DNA
30 binding sites showed that there was no large effect on the
gel shift species which formed after transgene induction
for at least 9 hours. Cotransfection of the blocker did
not interfere with the activity of a transcription factor
from a different family in cell culture. Considered

-73-

together, *hs-dCREB2-b* probably had fairly specific
molecular modes of action after induction.

Example 4   Assessment of the Role of CREBs in Long-Term
                 Memory Formation

5   Flies were fed 35 mM cycloheximide (CXM) for 12-14
hours before, or for the 24-hr retention interval
immediately after, massed or spaced training (Figure 8).
Each of these CXM feeding regimens significantly reduced
one-day memory after spaced training but had no effect on
10   one-day memory after massed training (Figure 8). Thus,
cyclohexmide feeding immediately before or after spaced
training disrupts one-day memory. These results suggest
that protein synthesis is required soon after training for
the formation of long-lasting memory.
15   The results in Figure 8 show that cycloheximide
feeding affects one-day retention after spaced training but
not massed training. Different groups of wild-type (Can-S)
flies were fed 5% glucose solution alone (hatched bars) or
laced with 35 mM CXM (striped bars) either for 12-14 hr
20   overnight before massed or spaced training or for the 24-hr
retention interval immediately after training. One-day
memory retention was significantly lower than normal in
flies fed CXM before (P < 0.001) or after (P < 0.001)
spaced training. In both cases, one-day retention in CXM-
25   fed flies was reduced to a level similar to one-day memory
after massed training in glucose-fed flies (P = 0.88 for
CXM before training and P = 0.71 for CXM after training).
In contrast, no difference was detected between CXM-fed and
control flies for one-day memory after massed training (P =
30   0.49 and P = 0.46, respectively).
         One day retention after spaced training was unaffected
in uninduced (-hs) transgenic flies *(17-2)* but was
significantly reduced in induced (+hs) transgenic flies
(Figure 9A). In contrast, one-day retention after massed

-74-

training was normal in both uninduced and induced transgenic flies (Figure 9A). Comparisons of one-day retention after spaced or massed training between wild-type flies with (+hs) or without (-hs) heat-shock indicated that

5   the heat-shock regimen itself did not have a non-specific effect on memory after either training protocol. Thus induction of the dCREB2-b transgene only affected (i.e., disrupted) one-day memory after spaced training.

One day retention after spaced or massed training in

10 M11-1, a second line carrying an independent hs-dCREB2-b insertion, also was tested. Results with M11-1 were similar to those obtained with 17-2 (Figure 9B). These results show that the effect of induced hs-dCREB2-b does not depend on any particular insertion site of the

15 transgene.

The results in Figures 9A-9C show that induction of the dCREB2-b transgene disrupts one-day memory after spaced training, while one-day memory after massed training and learning are normal.

20   In Figure 9A, different groups of wild-type (Can-S) flies (hatched bars) or hs-dCREB2-b transgenic (17-2) flies (striped bars) were given spaced training or massed training in the absence of heat shock (-hs) or three hours after heat shock (+hs). After training, flies were

25 transferred to standard food vials and stored at 18°C until one-day memory was assayed. No differences in one-day memory after spaced or massed training were detected between Can-S vs. 17-2 flies in the absence of heat shock (-hs; P = 0.83 and 0.63, respectively). When flies were

30 trained three hours after heat shock (+hs), however, one-day memory was significantly different between Can-S v. 17-2 flies after spaced training (P < 0.001) but not after massed training (P = 0.23). In fact, the one-day memory after spaced training was no different than that after

35 massed training in induced 17-2 flies (P = 0.59). In

-75-

addition, the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced (P = 0.59) or massed (P = 1.00) training in Can-S flies. N=6 performance indices (PIs) per group.

5   The experiment described in Figure 9A was repeated in Figure 9B with a second, independently derived dCREB2-b transgenic line, M11-1 (striped bars). Here again, a) no differences in one-day memory after spaced or massed training were detected between Can-S vs. M11-1 flies in the

10   absence of heat-shock (-hs; P = 0.83 and 0.86, respectively), b) a significant difference between Can-S v. M11-1 for one-day memory after spaced training (P < 0.001) but not after massed training (P = 0.85) when trained three hours after heat-shock (+hs), c) one-day memory after

15   spaced training was no different than that after massed training in induced M11-1 flies (P = 0.43) and d) the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced (P = 0.59) or massed (P = 0.94) training in Can-S flies. N=6 PIs per group.

20   If induction of the transgene specifically affected LTM via disruption of gene expression, then learning should not be affected, since it does not require new protein synthesis. Different groups of flies were trained using one-cycle training either without heat-shock, or three or

25   twenty four hours after heat-shock. These time points after induction were selected to correspond to the times when flies were trained and tested in the previous experiments (see Figures 9A and 9B). Induction of the transgene (d-CREB2-b) in the 17-2 line had no effect on

30   learning in either case (Figure 9C).

        In Figure 9C, different groups of Can-S flies (hatched bars) or 17-2 transgenic flies (striped bars) received one-cycle training in the absence of heat shock (-hs) or three (+hs 3hr) or 24 (+hs 24hr) hours after heat-shock and then

35   were tested immediately afterwards. In each case, no

-76-

differences between Can-S vs. 17-2 flies were detected (Ps = 0.28, 0.64 and 0.42, respectively), indicating that learning was normal in induced or uninduced transgenic flies. N=6 PIs per group.

5   Induction of the transgene which contained the mutant blocker (A2-2) did not affect one-day retention after spaced training, while the wild-type blocker (17-2) had a dramatic effect (Figure 10). The w(iso CJ1) flies, whose one-day retention also was unaffected by heat induction, is

10   the isogenic control for the mutant blocker transgenic flies. Since Western blot analysis showed that wild-type and mutant blockers probably have similar expression levels, this result suggests that the blocker requires an intact leucine zipper to function effectively.

15   Figure 10 shows that induction of the hs-dCREB2-mLZ mutant blocker does not affect one-day retention after spaced training. Different groups of wild-type [w (iso CJ1)], hs-dCREB2-b transgenic (17-2) or mutant hs-dCREB2-mLZ transgenic flies (A2-2) received spaced training in the

20   absence of heat-shock (-hs) or three hours after heat-shock (+hs). The flies were then handled and tested as in Figure 9A. No differences in one-day memory after spaced training were detected between w(isoCJ1) vs. 17-2 flies or between w(isoCJ1) vs. A2-2 flies in the absence of heat shock (-hs;

25   P = 0.38 and 0.59, respectively). When flies were trained three hours after heat shock (+hs), however, one-day memory after spaced training was significantly different between w(isoCJ1) vs. 17-2 flies (P < 0.001) -- as in Figure 9A -- but was not different between w(isoCJ1) vs. A2-2 flies (P =

30   0.78). In addition, the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced training in w(isoCJ1) or A2-2 flies (P = 0.40 and P = 0.97, respectively. N=6 performance indices (PIs) per group.

        Olfactory acuity and shock reactivity are component

35   behaviors essential for flies to properly learn odor-shock

-77-

associations. The Table shows the scores for these
peripheral behaviors for *Can-S* versus *17-2* flies. With or
without heat-shock, olfactory acuity and shock reactivity
were normal in *17-2* transgenic flies. Thus, *hs-dCREB2-b*
5   induction does not affect olfactory acuity or shock
reactivity.

    If induction of *hs-dCREB2-b* blocks long-term memory
(LTM), then long-lasting memory also should be blocked. In
wild-type flies, seven-day retention after spaced training
10 consists solely of the CXM-sensitive LTM because the CXM
insensitive ARM component has decayed away. In uninduced
transgenic flies (17-2), seven-day retention after spaced
training was similar to retention in uninduced wild-type
flies (P = 0.83; Figure 11). Seven-day retention was
15 severely disrupted, however, in transgenic flies which were
trained three hours after heat-shock (P = 0.001) and did
not differ from zero (P = 0.91). In contrast, the heat-
shock protocol had no detectable effect on seven-day memory
in wild-type flies (P = 0.39). Thus, induction of
20 *hs-dCREB2-b* disrupts long-term memory (LTM).

    Figure 11 shows that induction of *hs-dCREB2-b*
completely abolishes 7-day memory retention. Previous
analyses of *radish* mutants indicated that memory retention
four or more days after spaced training reflects the sole
25 presence of LTM. Thus, the effect of induced *hs-dCREB2-b*
on LTM was verified by comparing 7-day retention after
spaced training in Can-S (hatched bars) vs. *17-2* transgenic
(striped bars) flies that were trained in the absence of
heat-shock (-hs) or three hours after heat shock (+hs).
30 Flies were stored in standard food vials at 18°C during the
retention interval. N=6 PIs per group. Seven-day
retention after spaced training did not differ between Can-
S and *17-2* in the absence of heat-shock (P = 0.83) but was
significantly lower than normal in *17-2* flies after heat-
35 shock (P = 0.002). In fact, 7-day retention after spaced

-78-

training in induced 17-2 transgenic files was not different
from zero (P = 0.92). In addition, the heat-shock regimen
did not affect 7-day retention after spaced training non-
specifically in Can-S flies (P = 0.39).

5   If induction of the hs-dCREB2-b transgene specifically
blocks LTM, then it should only affect the CXM-sensitive
component of consolidated memory after spaced training.
For both transgenic lines, 17-2 and M11-1, the effect of
transgene induction looked similar to the effect that CXM
10   had on wild-type flies (compare Figure 8 with Figures 9A
and 9B). This similarity suggested that the induced
dCREB2-b protein completely blocked CXM-sensitive memory,
leaving ARM intact. The radish mutation disrupts ARM
(Folkers, E., et al., Proc.Natl.Acad.Sci. USA, 90: 8123-
15   8127 (1993)), leaving only LTM one day after spaced
training. Thus, a radish hs-dCREB2-b "double mutant" (rsh;
17-2) was constructed to allow examination of LTM in the
absence of ARM. In the absence of heat-shock, rsh;17-2
double-mutants and radish single-gene mutants yielded
20   equivalent one-day retention after spaced training (Figure
12). In contrast, when these flies were heat-shocked three
hours before spaced training, one-day retention was
undetectable in rsh;17-2 flies but remained at mutant
levels in radish flies. The double mutant also showed
25   normal (radish-like) learning (P = 0.59) and normal
(wild-type) olfactory acuity and shock reactivity in the
absence of heat-shock versus three hours after heat shock
(see the Table).
         Figure 12 shows that induction of hs-dCREB2-b
30   completely abolishes one-day memory after spaced training
in radish; 17-2 "double mutants." Since radish is known to
disrupt ARM, a clear view of the effect of hs-dCREB2-b on
LTM was obtained in radish;17-2 flies. One-day retention
after spaced training was assayed in rsh;17-2 double
35   mutants and in 17-2 and rsh single-gene mutants as

-79-

controls. Flies were trained in the absence of heat-shock (hatched bars) or three hours after heat-shock (striped bars) and stored at 18°C during the retention interval. As usual, induction of *hs-dCREB2-b* produced significantly
5 lower one-day memory after spaced training in *17-2* flies ($P < 0.001$). The heat-shock regimen, however, had no effect on such memory in *radish* mutants

TABLE 1.   Olfactory acuity and shock reactivity in Can-S (wild-type), 17-2 (hs-dCREB2-b transgenic) and rsh; 17-2 (radish, hs-dCREB2-b "double mutant") flies[a].

| Heat Shock | Group | Olfactory Acuity | | | | Shock Reactivity | |
| | | OCT | | MCH | | | |
		10^0	10^{-2}	10^0	10^{-2}	60V	20V
-hs	CAN-S	58±3	32±3	80±2	33±7	79±5	52±5
	17-2	60±3	34±8	77±3	37±5	87±3	43±2
+hs (3 hrs)	CAN-S	69±4	41±4	77±2	25±9	74±5	58±6
	17-2	71±4	37±3	76±5	26±3	78±3	67±5
+hs (24 hr)	CAN-S	66±2	56±8	79±4	33±2	84±3	63±3
	17-2	65±3	42±6	76±3	41±5	85±2	60±6
-hs	CAN-S	51±4	39±5	72±5	33±7	87±3	52±5
	rsh; 17-2	57±3	39±5	74±5	29±4	82±4	53±6
+hs (3 hr)	CAN-S	72±4	48±3	66±2	60±3	80±4	58±6
	rsh; 17-2	68±4	46±6	78±2	49±4	83±1	50±5

a   Olfactory acuity and shock reactivity were assayed in untrained flies with the methods of Boynton[1], S. and T. Tully, Genetics, 131: 655-672 (1992) and Dura, J-M., et al., J. Neurogenet., 9: 1-14 (1993), respectively (see Examples for more details). N=98 PIs per group. Planned comparisons between Can-S vs. mutant flies failed to detect any significant differences with any heat-shock regimen.

`10^0 is manual concentration and corresponds to 10^{-3} for bubbler.

-81-

(P = 0.52), which reflects only the presence of LTM. In contrast, heat-shock produced significantly lower scores in *rash;17-2* double mutants (P < 0.001), which were not different from zero (P = 0.20). N=6 PIs per group.

The following materials and methods were used in the work described in Examples 5 and 6.

Pavlovian Learning and Memory and Testing

During one training session, a group of about 100 flies was exposed sequentially to two odors [either octanol (OCT) or methylcyclohexanol (MCH)] for 60 seconds with 45-second rest intervals after each odor presentation. During exposure to the first odor, flies received twelve 1.5-second pulses of 60 V DC with a 5-second interpulse interval.

After training, flies were transferred to food vials and stored at 18°C for a seven-day retention interval. Conditioned odor-avoidance responses then were tested by transferring files to the choice point of a T-maze, where they were exposed simultaneously to OCT and MCH carried on converging currents of air in the distal ends of the T-maze arms and out the choice point.

Flies were allowed to distribute themselves in the T-maze arms for 120s, after which they were trapped in their respective arms, anesthetized and counted. The "percent correct" then was calculated as the number of flies avoiding the shock-paired odor (they were in the opposite T-maze arm) divided by the total number of flies in both arms. (The number of flies left at the choice point, which usually was less than 5%, were not included in this calculation.) Finally, a performance index (PI) was calculated by averaging the percent corrects of two reciprocal groups of flies -- one where OCT and shock were paired, the other where MCH and shock were paired--and then

-82-

by normalizing the average so that a PI=0 represented a
50:50 distribution in the T-maze and a PI=100 represented
100% avoidance of the shock-paired odor.

All behavioral experiments were designed in a balanced
5 fashion with N=2 PIs per group collected per day; then
replicated across days to generate final Ns. In all
experiments, the experimenter was blind to genotype.

Statistical Analyses of Behavior Data

10   PIs are distributed normally (Tully, T. and D. Gold,
J. Neurogenet., 9: 55-71 (1993)). Consequently,
untransformed (raw) data were analyzed parametrically with
JMP3.01 statistical software (SAS Institute Inc., Cary NC).
Negative accelerating exponential Gompertz (growth)
15 functions (see Lewis, D., Quantitative Methods in
Psychology, McGraw-Hill, New York, New York (1960)) were
fit to the data in Figures 13A and 13B via nonlinear least
squares with iteration.

20 Example 5   Effect on Long Term Memory of Repeated
                   Training Sessions
           Seven-day memory retention (a measure of long term
memory) in wild-type (Can-S) flies is induced incrementally
by repeated training sessions. An automated version of a
25 discriminative classical conditioning procedure was used to
electroshock flies during exposure to one odor (CS+) but
not to a second odor (CS-). Seven days after one or more
training sessions, memory retention of conditioned odor
avoidance responses was quantified in a T-maze, where flies
30 were presented the CS+ and CS- simultaneously for 120
seconds.
           In Figure 13A, long term memory as a function of the
number of training sessions is indicated by open circles.
One training session produced a mean performance index
35 (PI±SEM; Note 1) near zero. Additional training sessions

-83-

with a 15-minute rest interval between each, however, yielded a steady increase in mean PIs with a maximum of 39 after ten training sessions. Ten additional training sessions produced similar performance. A nonlinear
5   "growth" function (solid line) was fit to the individual PIs using an iterative least squares method. N = 13, 6, 6, 6, 13, 7, 7, 7, 7, 6, 7 and 7 PIs for groups receiving 1-10, 15 and 20 training sessions, respectively.

10   Example 6   Effect on Long Term Memory of the Rest
                        Interval Between Each Training Session
        Seven-day memory retention (a measure of long term memory) in wild-type (Can-S) flies is induced incrementally by the rest interval between each training session. As
15   described in Example 5, an automated version of a discriminative classical conditioning procedure was used to electroshock flies during exposure to one odor (CS+) but not to a second odor (CS-). Seven days after one or more training sessions, memory retention of conditioned odor
20   avoidance responses was quantified in a T-maze, where flies were presented the CS+ and CS- simultaneously for 120 seconds.
        In Figure 13B, long term memory as a function of the rest interval is indicated by open circles. Ten training
25   sessions with no rest interval between each (massed training) produced a mean PI near zero. Increasing the rest interval between each of ten training sessions yielded a steady increase in mean PIs with a maximum of 34 for a 10-minute rest interval. Rest intervals up to ten minutes
30   longer produced similar performance. A nonlinear growth function (solid line) was fit to the data as above. N = 12, 6, 6, 6, 6, 13, 7, 7, 7, 7, 7, 7 and 7 PIs for groups receiving 0-10, 15 and 20 minutes of rest between each training session.
35

-84-

The following materials and methods were used in the work described in Examples 7-10.

Isolating Transgenic Flies

5   EcoRI restriction sites were added (using PCR) just 5' to the putative translation initiation site and just 3' to the translation termination site in the dCREB2-a cDNA. This fragment was sequenced and subcloned into CaSpeR hs43, a mini-white transformation vector which contains the hsp70
10   promoter, in the orientation so that the dCREB2-a open reading frame is regulated by the hsp70 promoter. Germ-line transformation was accomplished by injecting into isogenic w(isoCJ1) embryoes using standard techniques (Spradling, A.C. and G.M. Rubin, Science, 218: 341-347
15   (1982); Rubin, G.M. and A. Spradling, Science, 218: 348-353 (1982)). By injecting DNA into the relatively homogeneous genetic background of w(isoCJ1), outcrossing of the resulting germ-line transformants to equilibrate (heterogeneous) genetic backgrounds was not necessary. Two
20   transgenic lines, C28 and C30, each with one independent P-element insertion were generated and characterized. They appeared normal in general appearance, fertility and viability. Flies homozygous for the C28 or C30 transgene were bred and used for all experiments.
25

Heat Shock Regimen

For heat-shock induction, flies were collected within two days of eclosion, placed in glass bottles in groups of about 600, and incubated overnight at 25°C and 70% relative
30   humidity. The next day, three hours before training, groups of approximately 100 flies were transferred to foam-stoppered glass shell vials containing a strip of filter paper to absorb excess moisture. The vials then were submerged in a 37°C water bath until the bottom of the foam
35   stopper (inside the vial) was below the surface of the

-85-

water, thereby insuring that the flies could not escape
heat-shock. The vial remained submerged for 30 minutes,
after which the flies were transferred to a standard food
vial for a 3-hr recovery period at 25°C and 70% relative
5   humidity. Training began immediately after the recovery
period.

Statistical Analyses of Behavior Data

PIs from the three strains (Can-S, C28 and C30) and
10 six training-regiments (1x+hs, 2xmassed+hs, 10xmassed+hs,
1x-hs, 2xmassed-hs and 10xmassed-hs) were subjected to a
TWO-WAY ANOVA with STRAIN ($F_{(2, 102)}=48.34$; P <0.001) and
TRAINing-regimen ($F_{(5, 102)}=25.47$, P <0.001) as main effects
and STRAIN x TRAIN ($F_{(10, 102)}=5.85$, P <0.001) as the
15 interaction term. Since preliminary experiments preceded
all of the experiments summarized herein, all pairwise
comparisons were planned. To maintain an experimentwise
error rate of alpha = 0.05, the individual comparisons
summarized in Figure 15B were judged significant if P <
20 0.002 (Sokal, R.R. and F.J. Rohlf, Biometry, 2nd Edition,
W.H. Freeman and Company, New York (1981)).

Example 7   A Molecular Switch for the Formation of Long
                Term Memory
25   Figure 14 presents a conceptual method of a molecular
switch for the formation of LTM, based on differential
regulation of CREB isoforms with opposing functions.
        Immediately after one training session, the relevant
CREB activators and repressors are induced. Their combined
30 functions (rather than molecular concentrations) are
equivalent and yielded no net effect of CREB activators.
Thus, repeated sessions of massed training (no rest
interval) never induce LTM (see Figure 15A). CREB
repressors functionally inactivate faster than CREB
35 activators, however, yielding an increasing net effect of

-86-

CREB activators (ΔC) with time (see Figure 13B). If ΔC is positive at the end of a particular rest interval during spaced training, then CREB activators are free to initiate downstream events involved with the formation of LTM.

5   Usually, ΔC after one training session is not large enough to yield much LTM. Thus, repeated spaced training sessions serve to increase ΔC incrementally eventually to produce maximal LTM (see Figure 13A).

Experimental verification of two predictions from this
10 model involving CREB as a molecular switch for long term memory formation is shown in Figures 15A-15C and discussed in Examples 8-10.

Example 8   Effect on Long Term Memory of Having Equal
15   Amounts of CREB Activators and Repressors
                   Immediately After One Training Session

The model of a molecular switch for LTM predicts that the functional effects of all CREB activators and repressors are equal immediately after one training session
20 (ΔC=0). If no rest interval occurred between additional training sessions (massed training), then functional CREB activator would not accumulate, thereby preventing the induction of downstream events required for LTM induction.

To test this notion, wild-type (Can-S) flies were
25 subjected to 48, instead of the usual 10 (see Figure 15B), massed training sessions (48x massed) or, as a positive control, to 10 spaced training sessions with a 15-minute rest interval (10x spaced). Seven-day memory after such massed training was near zero (Figure 15A), while that
30 after spaced training was near its usual maximum value (see Figure 13A). Thus, nearly five times the usual amount of massed training still did not induce LTM. N=6 PIs for each group.

PIs from two groups (10x spaced or 48x massed) of
35 wild-type (Can-S) files were subjected to a ONE-WAY ANOVA

-87-

with GROUP ($F_{(10)}=51.13$; P < 0.001) as the main effect. A subsequent planned comparison revealed that the mean PI of the 48x massed group did not differ significantly from zero ($t_{(10)}=1.66$; P=0.127).

5

Example 9   Effect on Long Term Memory of Increasing
                 Amounts of CREB Activator

The model of a molecular switch for LTM predicts that experimentally increasing the amount of CREB activator will
10   eliminate the requirements for at least 10 repeated training sessions with a 10-minute rest interval between each to produce maximal LTM.

To test this idea, two transgenic lines (C28 and C30) carrying an inducible hsp-dCREB2-a activator construct
15   inserted into different cytological locations were generated. Different groups of flies from these two transgenic lines were subjected, along with wild-type (Can-S) files, to 1 (1x) 2 (2x) or (10x) massed training sessions three hours after heat-shock induction of the
20   transgene (induced) or in the absence of heat-shock (uninduced).

Without heat-shock, seven-day memory in all three strains did not differ from zero after one, two or ten massed training sessions (all Ps > 0.002). With heat-
25   shock, seven-day memory in wild-type flies remained near zero in each massed training group (all Ps > 0.002). In contrast, seven-day memory was significant (near the maximum of 35) after ten massed sessions in both the C28 and C30 transgenic lines (all Ps < 0.0001). Moreover,
30   seven-day memory after one training session was similar to that after ten training sessions in both C28 (P = 0.89) and C30 (P = 0.89) transgenic flies. Thus, maximum LTM was induced after just one training session in transgenic flies expressing abnormally high levels of CREB activator. N=10,

-88-

4 and 6 PIs for each group of Can-S, *C28* and *C30*, respectively.

Example 10   Olfactory Acuity and Shock Reactivity

5   Odor avoidance responses to OCT or to MCH were quantified with the method of Boynton, S. and T. Tully, *Genetics*, *131*: 655-672 (1992), given a choice between an odor and air. The odors are naturally aversive, and flies usually chose air and avoided the T-maze arm containing the
10   odor. After 120 seconds, the flies were trapped in their respective arms of the T-maze, anesthetized and counted. A PI was calculated as a normalized percent correctly avoiding the odor (cf. Example 5). PIs from these two strains and two odor-groups (OCT and MCH) were subjected to
15   a TWO-WAY ANOVA with STRAIN $(F_{(1, 12)}=1.57$, $P=0.23)$ and ODOR $(F_{(1, 12)}=0.07$, $P=0.80)$ as main effects and DRUGxODOR $(F_{(1,12)}=0.15$, $P=0.71)$ as the interaction term. The two subsequent planned comparisons were judged significant if $P < 0.025$.
20   Shock reactivity was quantified with the method of Dura, J-M., et al., *J. Neurogenet.*, *9*: 1-14 (1993) in wild-type (Can-S) flies, or in a transgenic line (*C28*) carrying an inducible *hsp-dCREB2-a* construct, three hours after a 30-minute heat shock at 37°C. Briefly, flies were placed
25   in a T-maze and given a choice between an electrified grid (60V DC) in one T-maze arm and an unconnected grid in the other. After 120 seconds, the flies were trapped in their respective T-maze arms, anesthetized and counted. A PI was calculated as for olfactory acuity. PIs from these two
30   strains were subjected to a ONE- WAY ANOVA with STRAIN $(F_{(1, 6)}=13.03$, $P=0.01)$ as the main effect.
        Naive avoidance responses to odors or to shock three hours after heat-shock did not differ between wild-type (Can-S) versus transgenic (*C28*) flies for the two odorants
35   (MCH and OCT) used for conditioning experiments $(P=0.27$,

-89-

0.55, respectively). N=4 PIs per group. Naive shock
avoidance responses three hours after training for
transgenic flies were slightly lower than those for wild-
flies (P=0.01). N=4 PIs per group.

5

        Examples 11-13 pertain to the *Drosophila* nitric oxide
synthase work.

<u>Example 11</u>   <u>Low Stringency Hybridization to a Phage</u>
10   <u>Library of the *Drosophila* Genome and</u>
                        <u>Screening of *Drosophila* cDNA Library</u>
        $6x10^4$ plaques of a genomic *Drosophila* λDASH library
with the 1.3 kb Bgl II fragment of rat neuronal NOS cDNA
(residues 3282-4573) under low stringency conditions of 40%
15 formamide were screened as described in W.M. McGinnis *et
al.*, *Nature 308*: 428 (1984). Fifty positive phage were
purified and grouped based on *inter se* hybridization. The
contig containing the 2.4R fragment of *dNOS* was comprised
of 15 phage clones. Regions of cross-hybridization to the
20 rat probe were identified, subcloned and three of them were
sequenced. The other two did not contain sequences
homologous to any protein in the database. A *Drosophila*
head cDNA library (a gift from P. Salvaterra) was screened
with the 2.4R fragment isolated from phage clone λ8.11 in
25 standard conditions. All phage purification and cloning
steps were done with standard methods (J. Sambrook, E.F.
Fritsch, T. Maniatis, *Molecular cloning: A Laboratory
Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor,
NY, 1989)). cDNA fragments were subcloned into Bluescript
30 (Stratagene) and sequenced on both strands with Sequenase
2.0 (USB).

35

-90-

Example 12   Activity of _Drosophila_ Nitric Oxide Synthase
                (dNOS)

      The expression construct for activity assays contained
dNOS cDNA (with an _Xba_I site engineered immediately
5   upstream of the ATG codon) cloned into the _Xba_I and _Sma_I
    sites of the pCGN expression vector [M. Tanaka and W. Herr,
    Cell, _60_: 375 (1990)].   293 human kidney cells were
    transfected with 15μg of the _dNOS_ construct, or vector DNA,
    and precipitated with calcium phosphate as described in
10 [M.J. Imperiale, L.T. Feldman and J.R. Nevins, _Cell_, _35_:
    127 (1983)].   Cells were collected 2 days later and protein
    extracts were prepared as described in [J. Sambrook, E.F.
    Fritsch, T. Maniatis, _Molecular cloning: A Laboratory_
    Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor,
15 NY, 1989)].
      The fusion protein for raising anti-DNOS antibodies
    was made by cloning a 0.29 kb _Eam_1105I-_Sac_I fragment of
    dNOS cDNA (this fragment codes for 97 N-terminal amino
    acids of _dNOS_ ORF) into _Eco_RI site of pGEX-KG [K. Guan and
20 J.E. Dixon, _Anal. Biochem._, _192_: 262 (1991)].   The fusion
    protein was expressed in BL21 _E. coli_ strain and purified
    over Glutathione-Sepharose columns (Pharmacia) as described
    in [G.J. Hannon, D. Demetrick, D. Beach, _Genes & Dev._, _7_:
    2378 (1993)].   Immunization of rabbits, and serum
25 preparation, was done by Hazleton Research Products, Inc.
    (Denver).   The DNOS protein was detected on Western blots
    using a 1:500 dilution of rabbit serum, and cross-reacting
    bands were visualized with anti-rabbit alkaline phosphatase
    conjugate (Promega) according to the protocol provided.
30   The enzymatic assay was done essentially as described
    previously (D. Bredt and S. Snyder, _Proc. Natl. Acad. Sci._
    USA, _87_: 682 (1990)].   A 100 ml reaction mixture containing
    25 μl (50-100 μg) of soluble protein extract, 50 mM Hepes
    pH 7.4, 3 μM FAD, 3μM FMN, 10 μM tetrahydrobiopterin (ICN),
35 1 mM DTT, .8 mM $CaCl_2$, 1 mM NADPH, 10 μg/ml calmodulin, 2μl

-91-

of [³H]L-arginine (35.7 Ci/mmol, NEN) and 50 mM L-valine in was incubated for 60 minutes at 37°C. The reaction was stopped with 0.5 ml 20 mM Hepes pH 5.5, 2mM EDTA, 2mM EGTA, loaded on 0.5 ml Dowex AG 50WX-8 (Na⁺ form) column and
5 eluted with 3x0.5 ml of the stop buffer. Radioactivity present in the eluent was quantified in a scintillation counter.

Figures 17A-17B show the expression of DNOS enzymatic activity in 293 kidney cells. Figure 17A shows the results
10 of a Western blot analysis of protein extracts from 293 cells transfected with vector alone (lane 293 + vector) or with *dNOS* cDNA construct (lane 293 + dNOS). 25 μg of soluble protein extracts was resolved on 7.5% polyacrylamide gel, transferred to nitrocellulose membrane
15 and treated with anti-DNOS antibody. The arrow indicates the position of the DNOS protein. Positions of molecular weight markers (in kD) are shown on the left.

Figure 17B shows siginificant DNOS enzyme activity measured in 293 cell extracts by conversion of ³H-L-
20 arginine to ³H-L-citrulline. Enzymatic activity was detected only in cells transfected with *dNOS* cDNA construct (groups B-D) and is presented as specific activity (pmol of citrulline/mg/min.). The DNOS activity also was measured in the presence of 1 mM EGTA without exogenous Ca²⁺ or
25 calmodulin (group C), or in the presence of 100 mM L-NAME (group D). N=4 reactions per group.

Example 13   Splicing Pattern of dNOS
Heads and bodies of adult flies were separated on
30 sieves. Total RNA was isolated by the guanidinium isothiocyanate method [P. Chomczynski and N. Sacchi, *Anal. Biochem.*, *162*: 156 (1987)]. Poly(A)⁺ RNA selection, Northern blot and hybridization were done with standard methods (J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular*
35 *cloning: A Laboratory Manual* (Cold Spring Harbor

-92-

Laboratory, Cold Spring Harbor, NY, 1989)]. The blot was
hybridized with random-primed dNOS cDNA (10^6 cpm/ml), washed
in 0.1xSSC and 0.1% SDS at 65°C and exposed to X-ray film
for 72 hours. Two 25-mer primers [corresponding to

5   residues 1374-1399 (the top primer) and 1793-1817 (the
bottom primer) in the dNOS sequence] were used to amplify
fragments of two dNOS splice products. Each RT-PCR
reaction contained 30 ng of poly(A)⁺ head RNA. In the
first stage (RT), 90 ng of the bottom primer and 5U of rTth

10 polymerase (Perkin-Elmers) were added and the mixture was
incubated in the MJ Research Minicycler⁻ in the following
sequence of conditions: 95°C/1 minute, 67°C/45 seconds,
70°C/13 minutes. The second stage (PCR) was carried out as
follows: 94°C/45 seconds, 63°C/45 seconds, 70°C/90 seconds

15 and was repeated for 35 cycles. Products of the reaction
were analyzed on a denaturing polyacrylamide (8%) gel.
Bands of interest were isolated, reamplified, cloned into
pCR1000 (InVitrogen) and sequenced with Sequenase kit
(USB).

20   Northern blot analysis of dNOS expression in adult
flies shows a 5.0 kb dNOS transcript present in heads
(Figure 18A). Each lane contained 10 mg of poly (A)⁺ mRNA
isolated from Drosophila heads (H) or bodies (B). The
Northern blot was hybridized with the dNOS cDNA as

25 described above. Positions of size markers (in kb) are
shown on the left. The blot was overprobed with myosin
light chain (MLC) (Parker, V.P., Mol. Cell Biol. 5: 3058-
3068 (1985)) as a standard for RNA concentration.
         Figure 18b shows that the dNOS gene expresses two

30 alternatively spliced mRNA species. RT-PCR reactions were
performed on poly(A)⁺ mRNA isolated from Drosophila heads
and were resolved on 8% polyacrylamide gel. Arrows
indicate the positions of DNA fragments of expected sizes:
the 444 bp long-form fragment and the 129 bp short-form

35 fragment (lane +RNA). Other bands present in this lane are

-93-

artifacts from heteroduplexes that failed to denature.
Poly(A)$^+$ mRNA was omitted from the control reaction (lane
-RNA), which otherwise was done in identical conditions.
Size markers (kb ladder) are shown in the middle lane (KB).

5

Equivalents

Those skilled in the art will know, or be able to
ascertain, using no more than-routine experimentation, many
equivalents to the specific embodiments of the invention
10 described herein. These and all other equivalents are
intended to be encompassed by the following claims.

-94-

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        (A) NAME: Jerry Chi-Ping Yin
        (B) STREET: 47 Shady Lane
        (C) CITY: Huntington
        (D) STATE/PROVINCE: New York
        (E) COUNTRY: USA
        (F) POSTAL CODE/ZIP: 11743

        (A) NAME: Michael Regulski
        (B) STREET: 18 Hemlock Avenue
        (C) CITY: Huntington
        (D) STATE/PROVINCE: New York
        (E) COUNTRY: USA
        (F) POSTAL CODE/ZIP: 11743

    (ii) TITLE OF INVENTION: CLONING AND CHARACTERIZATION OF GENES
        ASSOCIATED WITH LONG-TERM MEMORY

    (iii) NUMBER OF SEQUENCES: 25

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.
        (B) STREET: Two Militia Drive
        (C) CITY: Lexington
        (D) STATE: Massachusetts
        (E) COUNTRY: USA
        (F) ZIP: 02173

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/361,063

-95-

(B) FILING DATE: 21-DEC-1994

(vii) PRIOR APPLICATION DATA:
    (A) APPLICATION NUMBER: US 08/319,866
    (B) FILING DATE: 07-OCT-1994

(viii) ATTORNEY/AGENT INFORMATION:
    (A) NAME: Granahan, Patricia
    (B) REGISTRATION NUMBER: 32,227
    (C) REFERENCE/DOCKET NUMBER: CSHL94-03A2 PCT

(ix) TELECOMMUNICATION INFORMATION:
    (A) TELEPHONE: (617) 861-6240
    (B) TELEFAX: (617) 861-9540

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1083 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "cDNA and PCR analysis"

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..1080

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GAC AAC AGC ATC GTC GAG GAG AAC GGC AAC TCG TCG GCG GCA TCG        48
Met Asp Asn Ser Ile Val Glu Glu Asn Gly Asn Ser Ser Ala Ala Ser
1               5                   10                  15

GGC TCC AAT GAC GTG GTC GAT GTC GTT GCC CAA CAG GCG GCG GCA GCG        96
Gly Ser Asn Asp Val Val Asp Val Val Ala Gln Gln Ala Ala Ala Ala
            20                  25                  30

GTG GGC GGC GGC GGT GGA GGA GGA GGA GGC GGC GGC GGC GGT GGT AAC       144
Val Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Asn
        35                  40                  45

CCC CAG CAG CAG CAA CAG AAC CCA CAA AGT ACA ACG GCC GGC GGT CCA       192
Pro Gln Gln Gln Gln Gln Asn Pro Gln Ser Thr Thr Ala Gly Gly Pro
    50                  55                  60

ACG GGT GCG ACG AAC AAC GCC CAG GGA GGC GGA GTG TCC TCC GTG CTG       240
Thr Gly Ala Thr Asn Asn Ala Gln Gly Gly Gly Val Ser Ser Val Leu
65                  70                  75                  80

ACC ACC ACC GCC AAC TGC AAC ATA CAA TAC CCC ATC CAG ACG CTG GCG       288
Thr Thr Thr Ala Asn Cys Asn Ile Gln Tyr Pro Ile Gln Thr Leu Ala
                85                  90                  95

CAG CAC GGA CTG CAG GTG AGC ATT TGG GGA CCG GGT GCT TGG TGT CAA       336
Gln His Gly Leu Gln Val Ser Ile Trp Gly Pro Gly Ala Trp Cys Gln
            100                 105                 110
```

-96-

```
CTG TCG AGT GTC AGG TGT TAC GGA TCC CAG CCA GAA GTG GCT ACC AAG        384
Leu Ser Ser Val Arg Cys Tyr Gly Ser Gln Pro Glu Val Ala Thr Lys
        115                 120                 125

GAT GTG CAG TCC GTG ATA CAG GCC AAT CCC TCG GGA GTC ATA CAG ACA        432
Asp Val Gln Ser Val Ile Gln Ala Asn Pro Ser Gly Val Ile Gln Thr
130                 135                 140

GCA GCT GGA ACC CAG CAG CAG CAA CAG GCG CTG GCC GCC GCC ACA GCG        480
Ala Ala Gly Thr Gln Gln Gln Gln Gln Ala Leu Ala Ala Ala Thr Ala
145                 150                 155                 160

ATG CAG AAG GTG GTC TAC GTG GCC AAG CCG CCG AAC TCG ACG GTC ATC        528
Met Gln Lys Val Val Tyr Val Ala Lys Pro Pro Asn Ser Thr Val Ile
                165                 170                 175

CAC ACG ACG CCT GGC AAT GCA GTG CAA GTG CGT AAC AAA ATC CCT CCA        576
His Thr Thr Pro Gly Asn Ala Val Gln Val Arg Asn Lys Ile Pro Pro
                180                 185                 190

ACC TTT CCA TGT AAG ATC AAG CCC GAA CCG AAC ACG CAG CAC CCG GAG        624
Thr Phe Pro Cys Lys Ile Lys Pro Glu Pro Asn Thr Gln His Pro Glu
                195                 200                 205

GAC AGC GAC GAG AGT CTG TCG GAC GAC GAT TCC CAG CAC CAC CGC AGC        672
Asp Ser Asp Glu Ser Leu Ser Asp Asp Asp Ser Gln His His Arg Ser
        210                 215                 220

GAG CTG ACG CGA CGG CCG TCG TAC AAT AAG ATC TTC ACC GAG ATC AGC        720
Glu Leu Thr Arg Arg Pro Ser Tyr Asn Lys Ile Phe Thr Glu Ile Ser
225                 230                 235                 240

GGT CCG GAC ATG AGC GGC GCA TCG CTT CCC ATG TCC GAC GGC GTG CTC        768
Gly Pro Asp Met Ser Gly Ala Ser Leu Pro Met Ser Asp Gly Val Leu
                245                 250                 255

AAT TCC CAG CTG GTG GGG ACC GGA GCG GGG GGC AAT GCG GCG AAC AGC        816
Asn Ser Gln Leu Val Gly Thr Gly Ala Gly Gly Asn Ala Ala Asn Ser
                260                 265                 270

TCC CTG ATG CAA TTG GAT CCC ACG TAC TAC CTG TCC AAT CGG ATG TCC        864
Ser Leu Met Gln Leu Asp Pro Thr Tyr Tyr Leu Ser Asn Arg Met Ser
                275                 280                 285

TAC AAC ACC AAC AAC AGC GGG ATA GCG GAG GAT CAG ACC CGT AAG CGC        912
Tyr Asn Thr Asn Asn Ser Gly Ile Ala Glu Asp Gln Thr Arg Lys Arg
                290                 295                 300

GAG ATC CGG CTG CAG AAG AAC AGG GAG GCG GCG CGT GAG TGC CGG CGC        960
Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu Cys Arg Arg
305                 310                 315                 320

AAG AAG AAG GAG TAC ATC AAG TGC CTG GAG AAT CGA GTG GCG GTG CTA       1008
Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val Ala Val Leu
                325                 330                 335

GAG AAC CAA AAC AAA GCG CTC ATC GAG GAG CTG AAG TCG CTC AAG GAG       1056
Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser Leu Lys Glu
                340                 345                 350
```

-97-

```
CTC TAT TGT CAG ACC AAG AAC GAT TGA                                    1083
Leu Tyr Cys Gln Thr Lys Asn Asp
        355             360
```

(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 360 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asp Asn Ser Ile Val Glu Glu Asn Gly Asn Ser Ser Ala Ala Ser
 1               5                  10                  15

Gly Ser Asn Asp Val Val Asp Val Val Ala Gln Gln Ala Ala Ala Ala
            20              25                  30

Val Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Asn
        35                  40                  45

Pro Gln Gln Gln Gln Gln Asn Pro Gln Ser Thr Thr Ala Gly Gly Pro
    50                  55                  60

Thr Gly Ala Thr Asn Asn Ala Gln Gly Gly Gly Val Ser Ser Val Leu
65                  70                  75                  80

Thr Thr Thr Ala Asn Cys Asn Ile Gln Tyr Pro Ile Gln Thr Leu Ala
                85                  90                  95

Gln His Gly Leu Gln Val Ser Ile Trp Gly Pro Gly Ala Trp Cys Gln
            100                 105                 110

Leu Ser Ser Val Arg Cys Tyr Gly Ser Gln Pro Glu Val Ala Thr Lys
            115                 120                 125

Asp Val Gln Ser Val Ile Gln Ala Asn Pro Ser Gly Val Ile Gln Thr
    130                 135                 140

Ala Ala Gly Thr Gln Gln Gln Gln Ala Leu Ala Ala Ala Thr Ala
145                 150                 155                 160

Met Gln Lys Val Val Tyr Val Ala Lys Pro Pro Asn Ser Thr Val Ile
                165                 170                 175

His Thr Thr Pro Gly Asn Ala Val Gln Val Arg Asn Lys Ile Pro Pro
            180                 185                 190

Thr Phe Pro Cys Lys Ile Lys Pro Glu Pro Asn Thr Gln His Pro Glu
        195                 200                 205

Asp Ser Asp Glu Ser Leu Ser Asp Asp Asp Ser Gln His His Arg Ser
    210                 215                 220

Glu Leu Thr Arg Arg Pro Ser Tyr Asn Lys Ile Phe Thr Glu Ile Ser
225                 230                 235                 240
```

-98-

```
Gly Pro Asp Met Ser Gly Ala Ser Leu Pro Met Ser Asp Gly Val Leu
            245             250             255

Asn Ser Gln Leu Val Gly Thr Gly Ala Gly Gly Asn Ala Ala Asn Ser
            260             265             270

Ser Leu Met Gln Leu Asp Pro Thr Tyr Tyr Leu Ser Asn Arg Met Ser
            275             280             285

Tyr Asn Thr Asn Asn Ser Gly Ile Ala Glu Asp Gln Thr Arg Lys Arg
    290             295             300

Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu Cys Arg Arg
305             310             315             320

Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val Ala Val Leu
            325             330             335

Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser Leu Lys Glu
            340             345             350

Leu Tyr Cys Gln Thr Lys Asn Asp
            355             360
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Arg Lys Arg Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu
1               5               10              15

Cys Arg Arg Lys Lys Lys Glu Tyr Ile Lys Cys Leu Glu Asn Arg Val
            20              25              30

Ala Val Leu Glu Asn Gln Asn Lys Ala Leu Ile Glu Glu Leu Lys Ser
            35              40              45

Leu Lys Glu Leu Tyr Cys
            50
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

-99-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Arg Lys Arg Glu Val Arg Leu Met Lys Asn Arg Glu Ala Ala Arg Glu
1               5                   10                  15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val
            20              25                  30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Ala
        35                  40                  45

Leu Lys Asp Leu Tyr Cys
        50
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Arg Lys Arg Glu Leu Arg Leu Met Lys Asn Arg Glu Ala Ala Arg Glu
1               5                   10                  15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val
            20              25                  30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Ala
        35                  40                  45

Leu Lys Asp Leu Tyr Cys
        50
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Leu Lys Arg Glu Ile Arg Leu Met Lys Asn Arg Glu Ala Ala Arg Glu
1               5                   10                  15

Cys Arg Arg Lys Lys Lys Glu Tyr Val Lys Cys Leu Glu Asn Arg Val
            20              25                  30

Ala Val Leu Glu Asn Gln Asn Lys Thr Leu Ile Glu Glu Leu Lys Thr
        35                  40                  45
```

-100-

Leu Lys Asp Leu Tyr Ser
    50

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 798 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..798

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG TTA CTC GGA GAA AAT ATG TTT TCT ACT TTC ACA TCG TTA GAT GCT   48
Met Leu Leu Gly Glu Asn Met Phe Ser Thr Phe Thr Ser Leu Asp Ala
  1   5   10   15

GCT ACC GCT ACA ACC AAC ACC GGT GAA TTC TTA ATG AAT GAA TCT CCA   96
Ala Thr Ala Thr Thr Asn Thr Gly Glu Phe Leu Met Asn Glu Ser Pro
              20   25   30

AGG CAA GAA GCC GGT GAC TTA ATG TTG GAT AGT CTG GAT TTC AAC ATT   144
Arg Gln Glu Ala Gly Asp Leu Met Leu Asp Ser Leu Asp Phe Asn Ile
              35   40   45

ATG GGC GAA AAC CTG GCA GAT GAT TTC CAG ACC TCG GCT TCA CCA GCT   192
Met Gly Glu Asn Leu Ala Asp Asp Phe Gln Thr Ser Ala Ser Pro Ala
      50   55   60

TCG GAG GAC AAG ATG ACT CCT TTC GTT GTT GAT ACC AAT GTT TTT GAA   240
Ser Glu Asp Lys Met Thr Pro Phe Val Val Asp Thr Asn Val Phe Glu
 65   70   75   80

TCC GTC TTC AAG AAC ACC GAA GAT ACC CTT CTA GGA GAT ATC GAC AAT   288
Ser Val Phe Lys Asn Thr Glu Asp Thr Leu Leu Gly Asp Ile Asp Asn
                  85   90   95

GTT GGT ATT GTT GAC ACG GAG TTG AAG GAG ATG TTC GAT TTG GTT GAC   336
Val Gly Ile Val Asp Thr Glu Leu Lys Glu Met Phe Asp Leu Val Asp
              100   105   110

TCG GAA ATC AAT AAC GGC ACT CCT ATC AAG CAG GAA GAA AAG GAT GAT   384
Ser Glu Ile Asn Asn Gly Thr Pro Ile Lys Gln Glu Glu Lys Asp Asp
              115   120   125

TTG GAA TTT ACT TCA AGA TCC CAG TCC ACC TCA GCT CTC TTG TCG TCG   432
Leu Glu Phe Thr Ser Arg Ser Gln Ser Thr Ser Ala Leu Leu Ser Ser
      130   135   140

AAA TCG ACT TCT GCT TCT CCA GCT GAT GCT GCC GCT GCA TGT GCA AGT   480
Lys Ser Thr Ser Ala Ser Pro Ala Asp Ala Ala Ala Ala Cys Ala Ser
145   150   155   160

-101-

```
CCT TCG TCA TCG TCT TGT AAA AGA TCC TAT TCT TCT GCT CAG CTA GAA        528
Pro Ser Ser Ser Ser Cys Lys Arg Ser Tyr Ser Ser Ala Gln Leu Glu
                165             170             175

ACT ACG GGT TCG GAT GCT CCA AAG AAA GAT AAG CTG GGC TGC ACC CCT        576
Thr Thr Gly Ser Asp Ala Pro Lys Lys Asp Lys Leu Gly Cys Thr Pro
                180             185             190

TAC ACT AGA AAA CAG AGA AAC AAT CCA TTA CCT CCG GTC ATT CCA AAG        624
Tyr Thr Arg Lys Gln Arg Asn Asn Pro Leu Pro Pro Val Ile Pro Lys
                195             200             205

GGT CAG GAT GTT GCT TCT ATG AAA AGG GCA AGA AAC ACT GAG GCC GCA        672
Gly Gln Asp Val Ala Ser Met Lys Arg Ala Arg Asn Thr Glu Ala Ala
        210             215             220

AGA AGA TCA AGA GCC AGA AAA ATG GAA AGA ATG TCC CAA CTT GAA GAA        720
Arg Arg Ser Arg Ala Arg Lys Met Glu Arg Met Ser Gln Leu Glu Glu
225             230             235             240

AAG TGT CAA AGC TTG TTG AAG GAA AAC GAC GAC TTG AAA GCT CAA GTT        768
Lys Cys Gln Ser Leu Leu Lys Glu Asn Asp Asp Leu Lys Ala Gln Val
                245             250             255

CAA GCT TTG AAG AAA TTA CTT GGA CAA CAA                                798
Gln Ala Leu Lys Lys Leu Leu Gly Gln Gln
                260             265
```

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 266 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Leu Leu Gly Glu Asn Met Phe Ser Thr Phe Thr Ser Leu Asp Ala
1               5               10              15

Ala Thr Ala Thr Thr Asn Thr Gly Glu Phe Leu Met Asn Glu Ser Pro
                20              25              30

Arg Gln Glu Ala Gly Asp Leu Met Leu Asp Ser Leu Asp Phe Asn Ile
        35              40              45

Met Gly Glu Asn Leu Ala Asp Asp Phe Gln Thr Ser Ala Ser Pro Ala
    50              55              60

Ser Glu Asp Lys Met Thr Pro Phe Val Val Asp Thr Asn Val Phe Glu
65              70              75              80

Ser Val Phe Lys Asn Thr Glu Asp Thr Leu Leu Gly Asp Ile Asp Asn
                85              90              95

Val Gly Ile Val Asp Thr Glu Leu Lys Glu Met Phe Asp Leu Val Asp
        100             105             110
```

-102-

Ser Glu Ile Asn Asn Gly Thr Pro Ile Lys Gln Glu Glu Lys Asp Asp
        115   120   125

Leu Glu Phe Thr Ser Arg Ser Gln Ser Thr Ser Ala Leu Leu Ser Ser
        130   135   140

Lys Ser Thr Ser Ala Ser Pro Ala Asp Ala Ala Ala Ala Cys Ala Ser
145   150   155   160

Pro Ser Ser Ser Ser Cys Lys Arg Ser Tyr Ser Ser Ala Gln Leu Glu
                165   170   175

Thr Thr Gly Ser Asp Ala Pro Lys Lys Asp Lys Leu Gly Cys Thr Pro
            180   185   190

Tyr Thr Arg Lys Gln Arg Asn Asn Pro Leu Pro Pro Val Ile Pro Lys
        195   200   205

Gly Gln Asp Val Ala Ser Met Lys Arg Ala Arg Asn Thr Glu Ala Ala
    210   215   220

Arg Arg Ser Arg Ala Arg Lys Met Glu Arg Met Ser Gln Leu Glu Glu
225   230   235   240

Lys Cys Gln Ser Leu Leu Lys Glu Asn Asp Asp Leu Lys Ala Gln Val
                245   250   255

Gln Ala Leu Lys Lys Leu Leu Gly Gln Gln
                260   265

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1350 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Met Ser Gln His Phe Thr Ser Ile Phe Glu Asn Leu Arg Phe Val Thr
    1   5   10   15

    Ile Lys Arg Ala Thr Asn Ala Gln Gln Gln Gln Gln Gln Gln Gln Gln
                20   25   30

    Gln Gln Leu Gln Gln Gln Gln Gln Gln Leu Gln Gln Gln Lys Ala Gln
            35   40   45

    Thr Gln Gln Gln Asn Ser Arg Lys Ile Lys Thr Gln Ala Thr Pro Thr
        50   55   60

    Leu Asn Gly Asn Gly Leu Leu Ser Gly Asn Pro Asn Gly Gly Gly Gly
    65   70   75   80

    Asp Ser Ser Pro Ser His Glu Val Asp His Pro Gly Gly Ala Gln Gly
                    85   90   95

-103-

Ala Gln Ala Ala Gly Gly Leu Pro Ser Leu Ser Gly Thr Pro Leu Arg
            100   105   110

His His Lys Arg Ala Ser Ile Ser Thr Ala Ser Pro Pro Ile Arg Glu
            115   120   125

Arg Arg Gly Thr Asn Thr Ser Ile Val Val Glu Leu Asp Gly Ser Gly
    130   135   140

Ser Gly Ser Gly Ser Gly Gly Gly Val Gly Val Gly Gln Gly Ala
145   150   155   160

Gly Cys Pro Pro Ser Gly Ser Cys Thr Ala Ser Gly Lys Ser Ser Arg
                165   170   175

Glu Leu Ser Pro Ser Pro Lys Asn Gln Gln Gln Pro Arg Lys Met Ser
                180   185   190

Gln Asp Tyr Arg Ser Arg Ala Gly Ser Phe Met His Leu Asp Asp Glu
        195   200   205

Gly Arg Ser Leu Leu Met Arg Lys Pro Met Arg Leu Lys Asn Ile Glu
    210   215   220

Gly Arg Pro Glu Val Tyr Asp Thr Leu His Cys Lys Gly Arg Glu Ile
225   230   235   240

Leu Ser Cys Ser Lys Ala Thr Cys Thr Ser Ser Ile Met Asn Ile Gly
                245   250   255

Asn Ala Ala Val Glu Ala Arg Lys Ser Asp Leu Ile Leu Glu His Ala
                260   265   270

Lys Asp Phe Leu Glu Gln Tyr Phe Thr Ser Ile Lys Arg Thr Ser Cys
        275   280   285

Thr Ala His Glu Thr Arg Trp Lys Gln Val Arg Gln Ser Ile Glu Thr
290   295   300

Thr Gly His Tyr Gln Leu Thr Glu Thr Glu Leu Ile Tyr Gly Ala Lys
305   310   315   320

Leu Ala Trp Arg Asn Ser Ser Arg Cys Ile Gly Arg Ile Gln Trp Ser
            325   330   335

Lys Leu Gln Val Phe Asp Cys Arg Tyr Val Thr Thr Thr Ser Gly Met
            340   345   350

Phe Glu Ala Ile Cys Asn His Ile Lys Tyr Ala Thr Asn Lys Gly Asn
            355   360   365

Leu Arg Ser Ala Ile Thr Ile Phe Pro Gln Arg Thr Asp Ala Lys His
    370   375   380

Asp Tyr Arg Ile Trp Asn Asn Gln Leu Ile Ser Tyr Ala Gly Tyr Lys
385   390   395   400

Gln Ala Asp Gly Lys Ile Ile Gly Asp Pro Met Asn Val Glu Phe Thr
            405   410   415

Glu Val Cys Thr Lys Leu Gly Trp Lys Ser Lys Gly Ser Glu Trp Asp
            420   425   430

-104-

```
Ile Leu Pro Leu Val Val Ser Ala Asn Gly His Asp Pro Asp Tyr Phe
        435                 440             445

Asp Tyr Pro Pro Glu Leu Ile Leu Glu Val Pro Leu Thr His Pro Lys
    450                 455             460

Phe Glu Trp Phe Ser Asp Leu Gly Leu Arg Trp Tyr Ala Leu Pro Ala
465                 470             475                 480

Val Ser Ser Met Leu Phe Asp Val Gly Gly Ile Gln Phe Thr Ala Thr
                485             490                 495

Thr Phe Ser Gly Trp Tyr Met Ser Thr Glu Ile Gly Ser Arg Asn Leu
                500             505             510

Cys Asp Thr Asn Arg Arg Asn Met Leu Glu Thr Val Ala Leu Lys Met
        515             520             525

Gln Leu Asp Thr Arg Thr Pro Thr Ser Leu Trp Lys Asp Lys Ala Val
    530             535             540

Val Glu Met Asn Ile Ala Val Leu His Ser Tyr Gln Ser Arg Asn Val
545                 550             555             560

Thr Ile Val Asp His His Thr Ala Ser Glu Ser Phe Met Lys His Phe
                565             570             575

Glu Asn Glu Ser Lys Leu Arg Asn Gly Cys Pro Ala Asp Trp Ile Trp
                580             585             590

Ile Val Pro Pro Leu Ser Gly Ser Ile Thr Pro Val Phe His Gln Glu
        595             600             605

Met Ala Leu Tyr Tyr Leu Lys Pro Ser Phe Glu Tyr Gln Asp Pro Ala
    610             615             620

Trp Arg Thr His Val Trp Lys Lys Gly Arg Gly Glu Ser Lys Gly Lys
625             630             635                 640

Lys Pro Arg Arg Lys Phe Asn Phe Lys Gln Ile Ala Arg Ala Val Lys
                645             650                 655

 Phe Thr Ser Lys Leu Phe Gly Arg Ala Leu Ser Lys Arg Ile Lys Ala
            660             665             670

 Thr Val Leu Tyr Ala Thr Glu Thr Gly Lys Ser Glu Gln Tyr Ala Lys
            675             680             685

 Gln Leu Cys Glu Leu Leu Gly His Ala Phe Asn Ala Gln Ile Tyr Cys
        690             695             700

 Met Ser Asp Tyr Asp Ile Ser Ser Ile Glu His Glu Ala Leu Leu Ile
705             710             715                 720

 Val Val Ala Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu Asn Gly Glu
            725             730             735

 Leu Phe Ser Gln Glu Leu Tyr Ala Met Arg Val Gln Glu Ser Ser Glu
        740             745             750

 His Gly Leu Gln Asp Ser Ser Ile Gly Ser Ser Lys Ser Phe Met Lys
        755             760             765
```

-105-

Ala Ser Ser Arg Gln Glu Phe Met Lys Leu Pro Leu Gln Gln Val Lys
    770   775   780

Arg Ile Asp Arg Trp Asp Ser Leu Arg Gly Ser Thr Ser Asp Thr Phe
785   790   795   800

Thr Glu Glu Thr Phe Gly Pro Leu Ser Asn Val Arg Phe Ala Val Phe
                805   810   815

Ala Leu Gly Ser Ser Ala Tyr Pro Asn Phe Cys Ala Phe Gly Gln Tyr
            820   825   830

Val Asp Asn Ile Leu Gly Glu Leu Gly Gly Glu Arg Leu Leu Arg Val
        835   840   845

Ala Tyr Gly Asp Glu Met Cys Gly Gln Glu Gln Ser Phe Arg Lys Trp
    850   855   860

Ala Pro Glu Val Phe Lys Leu Ala Cys Glu Thr Phe Cys Leu Asp Pro
865   870   875   880

Glu Glu Ser Leu Ser Asp Ala Ser Leu Ala Leu Gln Asn Asp Ser Leu
            885   890   895

Thr Val Asn Thr Val Arg Leu Val Pro Ser Ala Asn Lys Gly Ser Leu
        900   905   910

Asp Ser Ser Leu Ser Lys Tyr His Asn Lys Lys Val His Cys Cys Lys
        915   920   925

Ala Lys Ala Lys Pro His Asn Leu Thr Arg Leu Ser Glu Gly Ala Lys
    930   935   940

Thr Thr Met Leu Leu Glu Ile Cys Ala Pro Gly Leu Glu Tyr Glu Pro
945   950   955   960

Gly Asp His Val Gly Ile Phe Pro Ala Asn Arg Thr Glu Leu Val Asp
            965   970   975

Gly Leu Leu Asn Arg Leu Val Gly Val Asp Asn Pro Asp Glu Val Leu
        980   985   990

Gln Leu Gln Leu Leu Lys Glu Lys Gln Thr Ser Asn Gly Ile Phe Lys
    995   1000   1005

Cys Trp Glu Pro His Asp Lys Ile Pro Pro Asp Thr Leu Arg Asn Leu
    1010   1015   1020

Leu Ala Arg Phe Phe Asp Leu Thr Thr Pro Pro Ser Arg Gln Leu Leu
1025   1030   1035   1040

Thr Leu Leu Ala Gly Phe Cys Glu Asp Thr Ala Asp Lys Glu Arg Leu
            1045   1050   1055

Glu Leu Leu Val Asn Asp Ser Ser Ala Tyr Glu Asp Trp Arg His Trp
        1060   1065   1070

Arg Leu Pro His Leu Leu Asp Val Leu Glu Glu Phe Pro Ser Cys Arg
        1075   1080   1085

Pro Pro Ala Pro Leu Leu Leu Ala Gln Leu Thr Pro Leu Gln Pro Arg
    1090   1095   1100

-106-

```
Phe Tyr Ser Ile Ser Ser Ser Pro Arg Arg Val Ser Asp Glu Ile His
1105              1110            1115              1120

Leu Thr Val Ala Ile Val Lys Tyr Arg Cys Glu Asp Gly Gln Gly Asp
              1125            1130            1135

Glu Arg Tyr Gly Val Cys Ser Asn Tyr Leu Ser Gly Leu Arg Ala Asp
            1140            1145            1150

Asp Glu Leu Phe Met Phe Val Arg Ser Ala Leu Gly Phe His Leu Pro
        1155            1160            1165

Ser Asp Arg Ser Arg Pro Ile Ile Leu Ile Gly Pro Gly Thr Gly Ile
        1170            1175   __        1180

Ala Pro Phe Arg Ser Phe Trp Gln Glu Phe Gln Val Leu Arg Asp Leu
1185            1190            1195            1200

Asp Pro Thr Ala Lys Leu Pro Lys Met Trp Leu Phe Phe Gly Cys Arg
            1205            1210            1215

Asn Arg Asp Val Asp Leu Tyr Ala Glu Glu Lys Ala Glu Leu Gln Lys
            1220            1225            1230

Asp Gln Ile Leu Asp Arg Val Phe Leu Ala Leu Ser Arg Glu Gln Ala
            1235            1240            1245

Ile Pro Lys Thr Tyr Val Gln Asp Leu Ile Glu Gln Glu Phe Asp Ser
    1250            1255            1260

Leu Tyr Gln Leu Ile Val Gln Glu Arg Gly His Ile Tyr Val Cys Gly
1265            1270            1275            1280

Asp Val Thr Met Ala Glu His Val Tyr Gln Thr Ile Arg Lys Cys Ile
            1285            1290            1295

Ala Gly Lys Glu Gln Lys Ser Glu Ala Glu Val Glu Thr Phe Leu Leu
            1300            1305            1310

Thr Leu Arg Asp Glu Ser Arg Tyr His Glu Asp Ile Phe Gly Ile Thr
    1315            1320            1325

Leu Arg Thr Ala Glu Ile His Thr Lys Ser Arg Ala Thr Ala Arg Ile
    1330            1335            1340

Arg Met Ala Ser Gln Pro
1345            1350
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1205 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS:
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Gly Asn Leu Lys Ser Val Gly Gln Glu Pro Gly Pro Pro Cys Gly
1               5               10              15

Leu Gly Leu Gly Leu Gly Leu Gly Leu Cys Gly Lys Gln Gly Pro Ala
            20              25              30

Ser Pro Ala Pro Glu Pro Ser Arg Ala Pro Ala Pro Ala Thr Pro His
        35              40              45

Ala Pro Asp His Ser Pro Ala Pro Asn Ser Pro Thr Leu Thr Arg Pro
    50              55              60

Pro Glu Gly Pro Lys Phe Pro Arg Val Lys Asn Trp Glu Leu Gly Ser
65              70              75              80

Ile Thr Tyr Asp Thr Leu Cys Ala Gln Ser Gln Gln Asp Gly Pro Cys
            85              90              95

Thr Pro Arg Arg Cys Leu Gly Ser Leu Val Leu Pro Arg Lys Leu Gln
        100             105             110

Thr Arg Pro Ser Pro Gly Pro Pro Pro Ala Glu Gln Leu Leu Ser Gln
        115             120             125

Ala Arg Asp Phe Ile Asn Gln Tyr Tyr Ser Ser Ile Lys Arg Ser Gly
    130             135             140

Ser Gln Ala His Glu Glu Arg Leu Gln Glu Val Glu Ala Glu Val Ala
145             150             155             160

Ser Thr Gly Thr Ile His Leu Arg Glu Ser Glu Leu Val Phe Gly Ala
            165             170             175

Lys Gln Ala Trp Arg Asn Ala Pro Arg Cys Val Gly Arg Ile Gln Trp
        180             185             190

Gly Lys Leu Gln Val Phe Asp Ala Arg Asp Cys Ser Ser Ala Gln Glu
    195             200             205

Met Phe Thr Tyr Ile Cys Asn His Ile Lys Tyr Ala Thr Asn Arg Gly
210             215             220

Asn Leu Arg Ser Ala Ile Thr Val Phe Pro Gln Arg Ala Pro Gly Arg
225             230             235             240

Gly Asp Phe Arg Ile Trp Asn Ser Gln Leu Val Arg Tyr Ala Gly Tyr
            245             250             255

Arg Gln Gln Asp Gly Ser Val Arg Gly Asp Pro Ala Asn Val Glu Ile
        260             265             270

Thr Glu Leu Cys Ile Gln His Gly Trp Thr Pro Gly Asn Gly Arg Phe
        275             280             285

Asp Val Leu Pro Leu Leu Leu Gln Ala Pro Asp Glu Ala Pro Glu Leu
    290             295             300

Phe Val Leu Pro Pro Glu Leu Val Leu Glu Val Pro Leu Gly Ala Pro
305             310             315             320
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-108-

His Thr Gly Val Val Arg Gly Pro Gly Leu Arg Trp Tyr Ala Leu Pro
            325   330   335

Ala Val Ser Asn Met Leu Leu Glu Ile Gly Gly Leu Glu Phe Ser Ala
            340   345   350

Ala Pro Phe Ser Gly Trp Tyr Met Ser Thr Glu Ile Gly Thr Arg Asn
            355   360   365

Leu Cys Asp Pro His Arg Tyr Asn Ile Leu Glu Asp Val Ala Val Cys
            370   375   380

Met Asp Leu Asp Thr Arg Thr Thr Ser Ser Leu Trp Lys Asp Lys Ala
385   390   395   400

Ala Val Glu Ile Asn Leu Ala Val Leu His Ser Phe Gln Leu Ala Lys
                405   410   415

Val Thr Ile Val Asp His His Ala Ala Thr Val Ser Phe Met Lys His
            420   425   430

Leu Asp Asn Glu Gln Lys Ala Arg Gly Gly Cys Pro Ala Asp Trp Ala
            435   440   445

Trp Ile Val Pro Pro Ile Tyr Gly Ser Leu Pro Pro Val Phe His Gln
            450   455   460

Glu Met Val Asn Tyr Ile Leu Ser Pro Ala Phe Arg Tyr Gln Pro Asp
465   470   475   480

Pro Trp Lys Gly Ser Ala Thr Lys Gly Ala Gly Ile Thr Arg Lys Lys
                485   490   495

Thr Phe Lys Glu Val Ala Asn Ala Val Lys Ile Ser Ala Ser Leu Met
            500   505   510

Gly Thr Leu Met Ala Lys Arg Val Lys Ala Thr Ile Leu Tyr Ala Ser
            515   520   525

Glu Thr Gly Arg Ala Gln Ser Tyr Ala Gln Gln Leu Gly Arg Leu Phe
            530   535   540

Arg Lys Ala Phe Asp Pro Arg Val Leu Cys Met Asp Glu Tyr Asp Val
545   550   555   560

Val Ser Leu Glu His Glu Ala Leu Val Leu Val Val Thr Ser Thr Phe
                565   570   575

Gly Asn Gly Asp Pro Pro Glu Asn Gly Glu Ser Phe Ala Ala Ala Leu
            580   585   590

Met Glu Met Ser Gly Pro Tyr Asn Ser Ser Pro Arg Pro Glu Gln His
            595   600   605

Lys Ser Tyr Lys Ile Arg Phe Asn Ser Val Ser Cys Ser Asp Pro Leu
            610   615   620

Val Ser Ser Trp Arg Arg Lys Arg Lys Glu Ser Ser Asn Thr Asp Ser
625   630   635   640

Ala Gly Ala Leu Gly Thr Leu Arg Phe Cys Val Phe Gly Leu Gly Ser
                645   650   655

-109-

```
Arg Ala Tyr Pro His Phe Cys Ala Phe Ala Arg Ala Val Asp Thr Arg
            660             665                 670

Leu Glu Glu Leu Gly Gly Glu Arg Leu Leu Gln Leu Gly Gln Gly Asp
            675             680                 685

Glu Leu Cys Gly Gln Glu Glu Ala Phe Arg Gly Trp Ala Lys Ala Ala
        690             695             700

Phe Gln Ala Ser Cys Glu Thr Phe Cys Val Gly Glu Glu Ala Lys Ala
705             710             715                     720

Ala Ala Gln Asp Ile Phe Ser Pro Lys Arg Ser Trp Lys Arg Gln Arg
                725             730                 735

Tyr Arg Leu Ser Ala Gln Ala Glu Gly Leu Gln Leu Leu Pro Gly Leu
            740             745                 750

Ile His Val His Arg Arg Lys Met Phe Gln Ala Thr Val Leu Ser Val
            755             760             765

Glu Asn Leu Gln Ser Ser Lys Ser Thr Arg Ala Thr Ile Leu Val Arg
    770             775             780

Leu Asp Thr Ala Gly Gln Glu Gly Leu Gln Tyr Gln Pro Gly Asp His
785             790             795                     800

Ile Gly Ile Ser Ala Pro Asn Arg Pro Gly Leu Val Glu Ala Leu Leu
                805             810             815

Ser Arg Val Glu Asp Pro Pro Pro Thr Glu Ser Val Ala Val Glu
            820             825             830

Gln Leu Glu Lys Gly Ser Pro Gly Gly Pro Pro Pro Ser Trp Val Arg
        835             840             845

Asp Pro Arg Leu Pro Pro Cys Thr Val Arg Gln Ala Leu Thr Phe Phe
    850             855             860

Leu Asp Ile Thr Ser Pro Pro Ser Pro Arg Leu Leu Arg Leu Leu Ser
  865             870             875                     880

Thr Leu Ala Glu Glu Pro Ser Glu Gln Gln Glu Leu Glu Thr Leu Ser
            885             890                 895

Gln Asp Pro Arg Arg Tyr Glu Glu Trp Lys Leu Val Arg Cys Pro Thr
            900             905             910

Leu Leu Glu Val Leu Glu Gln Phe Pro Ser Val Ala Leu Pro Ala Pro
        915             920             925

Leu Leu Leu Thr Gln Leu Pro Leu Leu Gln Pro Arg Tyr Tyr Ser Val
    930             935             940

Ser Ser Ala Pro Asn Ala His Pro Gly Glu Val His Leu Thr Val Ala
945             950             955                     960

Val Leu Ala Tyr Arg Thr Gln Asp Gly Leu Gly Pro Leu His Tyr Gly
            965             970             975

Val Cys Ser Thr Trp Leu Ser Gln Leu Lys Thr Gly Asp Pro Val Pro
            980             985             990
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-110-

Cys Phe Ile Arg Gly Ala Pro Ser Phe Arg Leu Pro Pro Asp Pro Tyr
        995   1000   1005

Val Pro Cys Ile Leu Val Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg
    1010   1015   1020

Gly Phe Trp Gln Glu Arg Leu His Asp Ile Glu Ser Lys Gly Leu Gln
1025   1030   1035   1040

Pro His Pro Met Thr Leu Val Phe Gly Cys Arg Cys Ser Gln Leu Asp
                1045   1050   1055

His Leu Tyr Arg Asp Glu Val Gln Asp Ala Gln Glu Arg Gly Val Phe
            1060   1065   1070

Gly Arg Val Leu Thr Ala Phe Ser Arg Glu Pro Asp Ser Pro Lys Thr
            1075   1080   1085

Tyr Val Gln Asp Ile Leu Arg Thr Glu Leu Ala Ala Glu Val His Arg
        1090   1095   1100

Val Leu Cys Leu Glu Arg Gly His Met Phe Val Cys Gly Asp Val Thr
1105   1110   1115   1120

Met Ala Thr Ser Val Leu Gln Thr Val Gln Arg Ile Leu Ala Thr Glu
                1125   1130   1135

Gly Asp Met Glu Leu Asp Glu Ala Gly Asp Val Ile Gly Val Leu Arg
            1140   1145   1150

Asp Gln Gln Arg Tyr His Glu Asp Ile Phe Gly Leu Thr Leu Arg Thr
            1155   1160   1165

Gln Glu Val Thr Ser Arg Ile Arg Thr Gln Ser Phe Ser Leu Gln Glu
    1170   1175   1180

Arg His Leu Arg Gly Ala Val Pro Trp Ala Phe Asp Pro Pro Gly Pro
1185   1190   1195   1200

Asp Thr Pro Gly Pro
                1205

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1429 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

    Met Glu Glu Asn Thr Phe Gly Val Gln Gln Ile Gln Pro Asn Val Ile
    1   5   10   15

    Ser Val Arg Leu Phe Lys Arg Lys Val Gly Gly Leu Gly Phe Leu Val
                20   25   30

-111-

Lys Glu Arg Val Ser Lys Pro Pro Val Ile Ile Ser Asp Leu Ile Arg
    35   40   45

Gly Gly Ala Ala Glu Gln Ser Gly Leu Ile Gln Ala Gly Asp Ile Ile
    50   55   60

Leu Ala Val Asn Asp Arg Pro Leu Val Asp Leu Ser Tyr Asp Ser Ala
65   70   75   80

Leu Glu Val Leu Arg Gly Ile Ala Ser Glu Thr His Val Val Leu Ile
          85   90   95

Leu Arg Gly Pro Glu Gly Phe Thr Thr His Leu Glu Thr Thr Phe Thr
        100   105   110

Gly Asp Gly Thr Pro Lys Thr Ile Arg Val Thr Gln Pro Leu Gly Pro
        115   120   125

Pro Thr Lys Ala Val Asp Leu Ser His Gln Pro Ser Ala Ser Lys Asp
    130   135   140

Gln Ser Leu Ala Val Asp Arg Val Thr Gly Leu Gly Asn Gly Pro Gln
145   150   155   160

His Ala Gln Gly His Gly Gln Gly Ala Gly Ser Val Ser Gln Ala Asn
        165   170   175

Gly Val Ala Ile Asp Pro Thr Met Lys Ser Thr Lys Ala Asn Leu Gln
        180   185   190

Asp Ile Gly Glu His Asp Glu Leu Leu Lys Glu Ile Glu Pro Val Leu
        195   200   205

Ser Ile Leu Asn Ser Gly Ser Lys Ala Thr Asn Arg Gly Gly Pro Ala
    210   215   220

Lys Ala Glu Met Lys Asp Thr Gly Ile Gln Val Asp Arg Asp Leu Asp
225   230   235   240

Gly Lys Ser His Lys Ala Pro Pro Leu Gly Gly Asp Asn Asp Arg Val
        245   250   255

Phe Asn Asp Leu Trp Gly Lys Asp Asn Val Pro Val Ile Leu Asn Asn
        260   265   270

Pro Tyr Ser Glu Lys Glu Gln Ser Pro Thr Ser Gly Lys Gln Ser Pro
        275   280   285

Thr Lys Asn Gly Ser Pro Ser Arg Cys Pro Arg Phe Leu Lys Val Lys
    290   295   300

Asn Trp Glu Thr Asp Val Val Leu Thr Asp Thr Leu His Leu Lys Ser
305   310   315   320

Thr Leu Glu Thr Gly Cys Thr Glu His Ile Cys Met Gly Ser Ile Met
        325   330   335

Leu Pro Ser Gln His Thr Arg Lys Pro Glu Asp Val Arg Thr Lys Asp
        340   345   350

Gln Leu Phe Pro Leu Ala Lys Glu Phe Leu Asp Gln Tyr Tyr Ser Ser
        355   360   365

-112-

Ile Lys Arg Phe Gly Ser Lys Ala His Met Asp Arg Leu Glu Glu Val
370   375   380

Asn Lys Glu Ile Glu Ser Thr Ser Thr Tyr Gln Leu Lys Asp Thr Glu
385   390   395   400

Leu Ile Tyr Gly Ala Lys His Ala Trp Arg Asn Ala Ser Arg Cys Val
                405   410   415

Gly Arg Ile Gln Trp Ser Lys Leu Gln Val Phe Asp Ala Arg Asp Cys
            420   425   430

Thr Thr Ala His Gly Met Phe Asn Tyr Ile Cys Asn His Val Lys Tyr
            435   440   445

Ala Thr Asn Lys Gly Asn Leu Arg Ser Ala Ile Thr Ile Phe Pro Gln
450   455   460

Arg Thr Asp Gly Lys His Asp Phe Arg Val Trp Asn Ser Gln Leu Ile
465   470   475   480

Arg Tyr Ala Gly Tyr Lys Gln Pro Asp Gly Ser Thr Leu Gly Asp Pro
                485   490   495

Ala Asn Val Gln Phe Thr Glu Ile Cys Ile Gln Gln Gly Trp Lys Ala
            500   505   510

Pro Arg Gly Arg Phe Asp Val Leu Pro Leu Leu Leu Gln Ala Asn Gly
            515   520   525

Asn Asp Pro Glu Leu Phe Gln Ile Pro Pro Glu Leu Val Leu Glu Val
        530   535   540

Pro Ile Arg His Pro Lys Phe Asp Trp Phe Lys Asp Leu Gly Leu Lys
545   550   555   560

Trp Tyr Gly Leu Pro Ala Val Ser Asn Met Leu Leu Glu Ile Gly Gly
            565   570   575

Leu Glu Phe Ser Ala Cys Pro Phe Ser Gly Trp Tyr Met Gly Thr Glu
            580   585   590

Ile Gly Val Arg Asp Tyr Cys Asp Asn Ser Arg Tyr Asn Ile Leu Glu
        595   600   605

Glu Val Ala Lys Lys Met Asp Leu Asp Met Arg Lys Thr Ser Ser Leu
610   615   620

Trp Lys Asp Gln Ala Leu Val Glu Ile Asn Ile Ala Val Leu Tyr Ser
625   630   635   640

Phe Gln Ser Asp Lys Val Thr Ile Val Asp His His Ser Ala Thr Glu
            645   650   655

Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg Cys Arg Gly Gly Cys
            660   665   670

Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met Ser Gly Ser Ile Thr
        675   680   685

Pro Val Phe His Gln Glu Met Leu Asn Tyr Arg Leu Thr Pro Ser Phe
        690   695   700

-113-

Glu Tyr Gln Pro Asp Pro Trp Asn Thr His Val Trp Lys Gly Thr Asn
705   710   715   720

Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu
                725   730   735

Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln Ala Met Ala Lys Arg
            740   745   750

Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr Gly Lys Ser Gln Ala
        755   760   765

Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His Ala Phe Asp Ala Lys
    770   775   780

Ala Met Ser Met Glu Glu Tyr Asp Ile Val His Leu Glu His Glu Ala
785   790   795   800

Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu
            805   810   815

Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu Met Arg His Pro Asn
        820   825   830

Ser Val Gln Glu Glu Arg Lys Ser Tyr Lys Val Arg Phe Asn Ser Val
        835   840   845

Ser Ser Tyr Ser Asp Ser Arg Lys Ser Ser Gly Asp Gly Pro Asp Leu
    850   855   860

Arg Asp Asn Phe Glu Ser Thr Gly Pro Leu Ala Asn Val Arg Phe Ser
865   870   875   880

Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His Phe Cys Ala Phe Gly
            885   890   895

His Ala Val Asp Thr Leu Leu Glu Glu Leu Gly Gly Glu Arg Ile Leu
        900   905   910

Lys Met Arg Glu Gly Asp Glu Leu Cys Gly Gln Glu Glu Ala Phe Arg
    915   920   925

Thr Trp Ala Lys Lys Val Phe Lys Ala Ala Cys Asp Val Phe Cys Val
930   935   940

Gly Asp Asp Val Asn Ile Glu Lys Pro Asn Asn Ser Leu Ile Ser Asn
945   950   955   960

Asp Arg Ser Trp Lys Arg Asn Lys Phe Arg Leu Thr Tyr Val Ala Glu
            965   970   975

Ala Pro Asp Leu Thr Gln Gly Leu Ser Asn Val His Lys Lys Arg Val
        980   985   990

Ser Ala Ala Arg Leu Leu Ser Arg Gln Asn Leu Gln Ser Pro Lys Phe
    995   1000   1005

Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr Asn Gly Asn Gln Glu
    1010   1015   1020

Leu Gln Tyr Gln Pro Gly Asp His Leu Gly Val Phe Pro Gly Asn His
1025   1030   1035   1040

-114-

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Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu Glu Asp Ala Pro Pro
            1045                1050                1055

Ala Asn His Val Val Lys Val Glu Met Leu Glu Glu Arg Asn Thr Ala
            1060                1065                1070

Leu Gly Val Ile Ser Asn Trp Lys Asp Glu Ser Arg Leu Pro Pro Cys
            1075                1080                1085

Thr Ile Phe Gln Ala Phe Lys Tyr Tyr Leu Asp Ile Thr Thr Pro Pro
            1090                1095                1100

Thr Pro Leu Gln Leu Gln Gln Phe Ala Ser Leu Ala Thr Asn Glu Lys
1105                1110                1115                1120

Glu Lys Gln Arg Leu Leu Val Leu Ser Lys Gly Leu Gln Glu Tyr Glu
            1125                1130                1135

Glu Trp Lys Trp Gly Lys Asn Pro Thr Met Val Glu Val Leu Glu Glu
            1140                1145                1150

Phe Pro Ser Ile Gln Met Pro Ala Thr Leu Leu Leu Thr Gln Leu Ser
            1155                1160                1165

Leu Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Pro Asp Met Tyr
            1170                1175                1180

Pro Asp Glu Val His Leu Thr Val Ala Ile Val Ser Tyr His Thr Arg
1185                1190                1195                1200

Asp Gly Glu Gly Pro Val His His Gly Val Cys Ser Ser Trp Leu Asn
            1205                1210                1215

Arg Ile Gln Ala Asp Asp Val Val Pro Cys Phe Val Arg Gly Ala Pro
            1220                1225                1230

Ser Phe His Leu Pro Arg Asn Pro Gln Val Pro Cys Ile Leu Val Gly
            1235                1240                1245

Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln Arg Gln
            1250                1255                1260

Phe Asp Ile Gln His Lys Gly Met Asn Pro Cys Pro Met Val Leu Val
1265                1270                1275                1280

Phe Gly Cys Arg Gln Ser Lys Ile Asp His Ile Tyr Arg Glu Glu Thr
            1285                1290                1295

Leu Gln Ala Lys Asn Lys Gly Val Phe Arg Glu Leu Tyr Thr Ala Tyr
            1300                1305                1310

Ser Arg Glu Pro Asp Arg Pro Lys Lys Tyr Val Gln Asp Val Leu Gln
            1315                1320                1325

Glu Gln Leu Ala Glu Ser Val Tyr Arg Ala Leu Lys Glu Gln Gly Gly
            1330                1335                1340

His Ile Tyr Val Cys Gly Asp Val Thr Met Ala Ala Asp Val Leu Lys
1345                1350                1355                1360

Ala Ile Gln Arg Ile Met Thr Gln Gln Gly Lys Leu Ser Glu Glu Asp
            1365                1370                1375
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-115-

Ala Gly Val Phe Ile Ser Arg Leu Arg Asp Asp Asn Arg Tyr His Glu
        1380   1385   1390

Asp Ile Phe Gly Val Thr Leu Arg Thr Tyr Glu Val Thr Asn Arg Leu
        1395   1400   1405

Arg Ser Glu Ser Ile Ala Phe Ile Glu Glu Ser Lys Lys Asp Ala Asp
        1410   1415   1420

Glu Val Phe Ser Ser
1425

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1144 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ala Cys Pro Trp Lys Phe Leu Phe Lys Val Lys Ser Tyr Gln Ser
1   5   10   15

Asp Leu Lys Glu Glu Lys Asp Ile Asn Asn Asn Val Lys Lys Thr Pro
        20   25   30

Cys Ala Val Leu Ser Pro Thr Ile Gln Asp Asp Pro Lys Ser His Gln
        35   40   45

Asn Gly Ser Pro Gln Leu Leu Thr Gly Thr Ala Gln Asn Val Pro Glu
        50   55   60

Ser Leu Asp Lys Leu His Val Thr Ser Thr Arg Pro Gln Tyr Val Arg
65   70   75   80

Ile Lys Asn Trp Gly Ser Gly Glu Ile Leu His Asp Thr Leu His His
                85   90   95

Lys Ala Thr Ser Asp Phe Thr Cys Lys Ser Lys Ser Cys Leu Gly Ser
            100   105   110

Ile Met Asn Pro Lys Ser Leu Thr Arg Gly Pro Arg Asp Lys Pro Thr
        115   120   125

Pro Leu Glu Glu Leu Leu Pro His Ala Ile Glu Phe Ile Asn Gln Tyr
    130   135   140

Tyr Gly Ser Phe Lys Glu Ala Lys Ile Glu Glu His Leu Ala Arg Leu
145   150   155   160

Glu Ala Val Thr Lys Glu Ile Glu Thr Thr Gly Thr Tyr Gln Leu Thr
            165   170   175

Leu Asp Glu Leu Ile Phe Ala Thr Lys Met Ala Trp Arg Asn Ala Pro
        180   185   190

-116-

Arg Cys Ile Gly Arg Ile Gln Trp Ser Asn Leu Gln Val Phe Asp Ala
    195   200   205

Arg Asn Cys Ser Thr Ala Gln Glu Met Phe Gln His Ile Cys Arg His
    210   215   220

Ile Leu Tyr Ala Thr Asn Asn Gly Asn Ile Arg Ser Ala Ile Thr Val
225   230   235   240

Phe Pro Gln Arg Ser Asp Gly Lys His Asp Phe Arg Leu Trp Asn Ser
                245   250   255

Gln Leu Ile Arg Tyr Ala Gly Tyr Gln Met Pro Asp Gly Thr Ile Arg
            260   265   270

Gly Asp Ala Ala Thr Leu Glu Phe Thr Gln Leu Cys Ile Asp Leu Gly
        275   280   285

Trp Lys Pro Arg Tyr Gly Arg Phe Asp Val Leu Pro Leu Val Leu Gln
    290   295   300

Ala Asp Gly Gln Asp Pro Glu Val Phe Glu Ile Pro Pro Asp Leu Val
305   310   315   320

Leu Glu Val Thr Met Glu His Pro Lys Tyr Glu Trp Phe Gln Glu Leu
                325   330   335

Gly Leu Lys Trp Tyr Ala Leu Pro Ala Val Ala Asn Met Leu Leu Glu
                340   345   350

Val Gly Gly Leu Glu Phe Pro Ala Cys Pro Phe Asn Gly Trp Tyr Met
            355   360   365

Gly Thr Glu Ile Gly Val Arg Asp Phe Cys Asp Thr Gln Arg Tyr Asn
    370   375   380

Ile Leu Glu Glu Val Gly Arg Arg Met Gly Leu Glu Thr His Thr Leu
385   390   395   400

Ala Ser Leu Trp Lys Asp Arg Ala Val Thr Glu Ile Asn Val Ala Val
                405   410   415

Leu His Ser Phe Gln Lys Gln Asn Val Thr Ile Met Asp His His Thr
            420   425   430

Ala Ser Glu Ser Phe Met Lys His Met Gln Asn Glu Tyr Arg Ala Arg
        435   440   445

Gly Gly Cys Pro Ala Asp Trp Ile Trp Leu Val Pro Pro Val Ser Gly
    450   455   460

Ser Ile Thr Pro Val Phe His Gln Glu Met Leu Asn Tyr Val Leu Ser
465   470   475   480

Pro Phe Tyr Tyr Tyr Gln Ile Glu Pro Trp Lys Thr His Ile Trp Gln
                485   490   495

Asn Glu Lys Leu Arg Pro Arg Arg Arg Glu Ile Arg Phe Arg Val Leu
            500   505   510

Val Lys Val Val Phe Phe Ala Ser Met Leu Met Arg Lys Val Met Ala
        515   520   525

-117-

Ser Arg Val Arg Ala Thr Val Leu Phe Ala Thr Glu Thr Gly Lys Ser
    530   535   540

Glu Ala Leu Ala Arg Asp Leu Ala Thr Leu Phe Ser Tyr Ala Phe Asn
545   550   555   560

Thr Lys Val Val Cys Met Asp Gln Tyr Lys Ala Ser Thr Leu Glu Glu
                565   570   575

Glu Gln Leu Leu Leu Val Val Thr Ser Thr Phe Gly Asn Gly Asp Cys
            580   585   590

Pro Ser Asn Gly Gln Thr Leu Lys Lys Ser Leu Phe Met Leu Arg Glu
            595   600   605

Leu Asn His Thr Phe Arg Tyr Ala Val Phe Gly Leu Gly Ser Ser Met
    610   615   620

Tyr Pro Gln Phe Cys Ala Phe Ala His Asp Ile Asp Gln Lys Leu Ser
625   630   635   640

His Leu Gly Ala Ser Gln Leu Ala Pro Thr Gly Glu Gly Asp Glu Leu
            645   650   655

Ser Gly Gln Glu Asp Ala Phe Arg Ser Trp Ala Val Gln Thr Phe Arg
            660   665   670

Ala Ala Cys Glu Thr Phe Asp Val Arg Ser Lys His His Ile Gln Ile
        675   680   685

Pro Lys Arg Phe Thr Ser Asn Ala Thr Trp Glu Pro Gln Gln Tyr Arg
    690   695   700

Leu Ile Gln Ser Pro Glu Pro Leu Asp Leu Asn Arg Ala Leu Ser Ser
705   710   715   720

Ile His Ala Lys Asn Val Phe Thr Met Arg Leu Lys Ser Gln Gln Asn
                725   730   735

Leu Gln Ser Glu Lys Ser Ser Arg Thr Thr Leu Leu Val Gln Leu Thr
            740   745   750

Phe Glu Gly Ser Arg Gly Pro Ser Tyr Leu Pro Gly Glu His Leu Gly
            755   760   765

Ile Phe Pro Gly Asn Gln Thr Ala Leu Val Gln Gly Ile Leu Glu Arg
    770   775   780

Val Val Asp Cys Pro Thr Pro His Gln Thr Val Cys Leu Glu Val Leu
785   790   795   800

Asp Glu Ser Gly Ser Tyr Trp Val Lys Asp Lys Arg Leu Pro Pro Cys
            805   810   815

Ser Leu Ser Gln Ala Leu Thr Tyr Phe Leu Asp Ile Thr Thr Pro Pro
            820   825   830

Thr Gln Leu Gln Leu His Lys Leu Ala Arg Phe Ala Thr Asp Glu Thr
    835   840   845

Asp Arg Gln Arg Leu Glu Ala Leu Cys Gln Pro Ser Glu Tyr Asn Asp
850   855   860

-118-

```
Trp Lys Phe Ser Asn Asn Pro Thr Phe Leu Glu Val Leu Glu Glu Phe
865                 870             875                 880

Pro Ser Leu His Val Pro Ala Ala Phe Leu Leu Ser Gln Leu Pro Ile
                885             890                 895

Leu Lys Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Gln Asp His Thr Pro
            900             905             910

Ser Glu Val His Leu Thr Val Ala Val Val Thr Tyr Arg Thr Arg Asp
            915             920             925

Gly Gln Gly Pro Leu His His Gly Val Cys Ser Thr Trp Ile Arg Asn
    930             935             940

Leu Lys Pro Gln Asp Pro Val Pro Cys Phe Val Arg Ser Val Ser Gly
945             950             955                 960

Phe Gln Leu Pro Glu Asp Pro Ser Gln Pro Cys Ile Leu Ile Gly Pro
            965             970             975

Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln Arg Leu His
            980             985             990

Asp Ser Gln His Lys Gly Leu Lys Gly Gly Arg Met Ser Leu Val Phe
    995             1000            1005

Gly Cys Arg His Pro Glu Glu Asp His Leu Tyr Gln Glu Glu Met Gln
    1010            1015            1020

Glu Met Val Arg Lys Arg Val Leu Phe Gln Val His Thr Gly Tyr Ser
1025            1030            1035            1040

Arg Leu Pro Gly Lys Pro Lys Val Tyr Val Gln Asp Ile Leu Gln Lys
            1045            1050            1055

Gln Leu Ala Asn Glu Val Leu Ser Val Leu His Gly Glu Gln Gly His
            1060            1065            1070

Leu Tyr Ile Cys Gly Asp Val Arg Met Ala Arg Asp Val Ala Thr Thr
            1075            1080            1085

Leu Lys Lys Leu Val Ala Thr Lys Leu Asn Leu Ser Glu Glu Gln Val
            1090            1095            1100

Glu Asp Tyr Phe Phe Gln Leu Lys Ser Gln Lys Arg Tyr His Glu Asp
    1105            1110            1115            1120

Ile Phe Gly Ala Val Phe Ser Tyr Gly Ala Lys Lys Gly Ser Ala Leu
            1125            1130            1135

Glu Glu Pro Lys Ala Thr Arg Leu
            1140
```

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

-119-

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Asp Pro Ala Asn Val Glu Phe Thr Glu Ile Cys Ile Gln Gln Gly
1   5   10   15

Trp Lys Pro Arg
            20

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Asp Pro Met Asn Val Glu Phe Thr Glu Thr Val Ala Leu Lys Met
1   5   10   15

Gln Leu Asp Thr
            20

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Cys Asp Asn Ser Arg Tyr Asn Ile Leu Glu Glu Val Ala Lys Lys Met
1   5   10   15

Asp Leu Asp Met
            20

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-120-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gly Asp Pro Ala Asn Val Glu Phe Thr Glu Glu Val Ala Lys Lys Met
1   5   10   15

Asp Leu Asp Met
          20

(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 57 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: double
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

_____

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGTCTAGATC TATGACTGAA TATGACGTAA TATGACGTAA TGGTACCAGA TCTGGCC   57

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 41 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: double
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAATGACGTA ACGGAAATGA CGTAACGGAA ATGACGTAAC G   41

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 42 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: double
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAATGAATTA ACGGAAATGA ATTAACGGAA ATGAATTAAC GG   42

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 81 base pairs
                (B) TYPE: nucleic acid

-121-

        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGCACGGGTT TTCGACGTTC ACTGGTAGTG TCTGATGAGG CCGAAAGGCC GAAACGCGAT   60

GCCCATAACC ACCACGCTCA G   81

(2) INFORMATION FOR SEQ ID NO:21:

   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 100 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGACCCACA GTTTCGGGTT TTCGAGCAAG TCTGCTAGTG TCTGATGAGG CCGAAAGGCC   60

GAAACGCGAA GCCGTATTGC ACCACGCTCA TCGAGAAGGC   100

(2) INFORMATION FOR SEQ ID NO:22:

   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 55 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGAGCTTG CAAGCATGCT TGCAAGCAAG CATGCTTGCA AGCATGCTTG CAAGC   55

(2) INFORMATION FOR SEQ ID NO:23:

   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCTAGAGCG TACGCAAGCG TACGCAAGCG TACG   34

-122-

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 17 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS:
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Arg Lys Arg Glu Ile Arg Leu Gln Lys Asn Arg Glu Ala Ala Arg Glu
1   5   10   15

Cys

---

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 4491 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
GAATTCCGTT TTTGAAAAGT GAAGCAATTG AGTGCGGCCC GAAAAAGAGA GCCGCAGAAA      60

GTTTGCGAAC AGAATTTAAT CAAAAACTTG GAGGGTAAAT TGTCCAAGTG GTTCACCTGT     120

TGGCTGCATT TTAAATCAAC GAGGCAAACA ATCAGCGCAG AGGAGCTGCT CCACGTTCCC     180

CGGACAAGAT GTCGCAGCAT TTCACATCGA TATTTGAGAA CCTGCGATTC GTGACCATCA     240

AACGTGCGAC AAATGCGCAA CAGCAACAGC AGCAGCAGCA GCAACAGCAA CTTCAGCAGC     300

AGCAGCAGCA GCTGCAGCAA CAGAAGGCAC AGACACAGCA ACAAAATAGC AGAAAAATCA     360

AAACTCAAGC AACGCCAACG TTGAATGGCA ATGGGCTCTT GAGCGGCAAT CCAAATGGCG     420

GAGGCGGTGA CTCCTCGCCC AGCCATGAAG TGGACCATCC GGGTGGAGCA CAAGGAGCTC     480

AAGCAGCAGG AGGCTTGCCA TCTTTAAGTG GCACGCCATT GAGGCACCAC AAGCGCGCCA     540

GTATCTCCAC AGCATCGCCT CCAATTCGCG AACGGCGTGG CACCAACACC AGCATCGTGG     600

TCGAACTGGA TGGCAGTGGC AGCGGGAGTG GGAGTGGCGG TGGTGGCGTT GGCGTTGGTC     660

AGGGTGCGGG TTGTCCTCCC TCGGGCAGCT GCACTGCGTC CGGAAAAAGT TCGCGGGAAC     720

TATCGCCGTC GCCGAAAAAC CAACAGCAGC CCAGAAAGAT GTCACAGGAT TATCGGTCGC     780

GTGCCGGCAG CTTTATGCAC CTGGACGACG AGGGACGCAG TCTGCTGATG CGCAAGCCGA     840
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-123-

```
TGAGACTGAA GAACATCGAG GGCAGGCCGG AGGTCTACGA CACGCTGCAC TGCAAGGGTC    900

GCGAGATTCT TTCCTGCTCG AAGGCCACCT GTACGAGCAG CATTATGAAC ATTGGCAATG    960

CGGCGGTGGA GGCCAGGAAA TCCGATCTGA TCCTCGAACA CGCCAAGGAC TTCCTCGAGC   1020

AGTACTTTAC ATCGATAAAG CGTACATCAT GTACCGCCCA CGAGACGCGA TGGAAACAGG   1080

TGCGCCAGAG CATTGAGACC ACTGGACACT ATCAGCTAAC CGAAACGGAG CTAATTTATG   1140

GTGCCAAATT GGCCTGGCGC AATTCTTCAC GTTGCATTGG CCGAATACAA TGGTCGAAGT   1200

TGCAGGTCTT TGACTGTCGT TATGTGACAA CAACAAGTGG CATGTTTGAA GCCATTTGCA   1260

ATCACATTAA ATATGCAACA AATAAGGGCA ACCTGAGATC GGCCATCACG ATATTTCCAC   1320

AACGCACAGA TGCCAAGCAT GATTATCGCA TTTGGAATAA CCAATTAATA TCTTATGCCG   1380

GCTACAAGCA GGCGGATGGA AAAATCATTG GCGATCCCAT GAATGTGGAG TTTACAGAGG   1440

TCTGCACCAA GCTGGGCTGG AAGAGCAAGG GCAGCGAGTG GGACATACTG CCATTGGTGG   1500

TCTCGGCCAA TGGTCACGAT CCGGACTACT TTGATTACCC GCCCGAATTG ATACTGGAAG   1560

TTCCGCTGAC CCATCCCAAA TTCGAATGGT CTCGGATCT GGGACTGCGA TGGTACGCCC    1620

TGCCCGCCGT ATCCAGTATG CTGTTCGATG TGGGCGGCAT TCAGTTTACG GCCACCACAT   1680

TCAGTGGTTG GTACATGTCG ACAGAGATTG GCAGCCGGAA TTTATGCGAC ACAAATCGCC   1740

GCAATATGCT GGAGACGGTG GCGCTGAAGA TGCAACTGGA CACCCGTACG CCCACATCCT   1800

TGTGGAAGGA CAAGGCTGTG GTGGAGATGA ACATTGCCGT GCTCCACTCC TACCAGAGTC   1860

GCAACGTGAC CATTGTGGAT CACCACACGG CCAGCGAGAG CTTTATGAAG CATTTCGAGA   1920

ACGAGTCCAA GCTCAGGAAT GGGTGTCCCG CTGATTGGAT TTGGATCGTG CCGCCGCTGT   1980

CGGGCTCCAT AACGCCGGTA TTCCATCAGG AGATGGCTCT GTACTACCTG AAGCCCTCGT   2040

TCGAGTACCA GGATCCCGCC TGGCGAACCC ACGTGTGGAA AAAGGGGCGT GGCGAGAGCA   2100

AGGGCAAGAA GCCAAGACGT AAATTCAATT TTAAACAAAT CGCTAGGGCT GTGAAATTTA   2160

CATCGAAACT ATTTGGACGC GCCTTATCGA AACGCATAAA GGCAACAGTT CTATATGCCA   2220

CCGAAACTGG CAAATCGGAG CAGTATGCGA AGCAACTTTG TGAACTCCTA GGGCACGCAT   2280

TCAATGCACA GATATATTGC ATGTCCGACT ACGATATATC CTCCATTGAG CACGAGGCAT   2340

TGTTAATTGT TGTGGCCTCC ACCTTTGGCA ACGGTGATCC CCCCGAAAAC GGCGAGCTTT   2400

TCTCCCAGGA ATTGTATGCG ATGCGTGTCC AGGAGTCTTC CGAGCATGGA TTGCAGGACT   2460

CCAGCATTGG CTCGTCAAAG TCCTTCATGA AGGCCAGCTC GCGGCAGGAG TTCATGAAGC   2520

TGCCACTGCA ACAGGTGAAG AGAATCGACC GATGGGACTC GCTGCGGGGC TCCACCTCGG   2580

ACACCTTCAC CGAGGAGACC TTTGGTCCCC TCTCCAATGT CCGGTTTGCC GTTTTTGCCC   2640

TCGGCTCCTC GGCCTATCCA AATTTCTGCG CCTTCGGTCA GTATGTGGAC AACATTCTGG   2700
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-124-

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GCGAGCTGGG CGGCGAACGC CTGCTGAGGG TGGCCTACGG CGACGAGATG TGCGGACAGG    2760

AGCAGTCGTT CCGGAAGTGG GCGCCCGAGG TATTCAAGTT GGCCTGCGAG ACCTTCTGCC    2820

TGGATCCAGA GGAGAGCCTT TCGGATGCCT CGCTAGCCCT GCAGAACGAT TCGCTGACTG    2880

TGAATACGGT GCGCCTGGTG CCGTCGGCGA ATAAGGGATC CCTGGACAGC AGTTTATCCA    2940

AGTACCACAA CAAGAAGGTG CACTGCTGCA AGGCGAAGGC GAAGCCCCAC AATTTGACCC    3000

GTTTGAGTGA GGGAGCCAAG ACAACGATGC TGCTGGAGAT CTGTGCACCT GGCTTGGAGT    3060

ACGAGCCGGG TGATCATGTG GGCATCTTTC CGGCGAATCG AACGGAACTG GTCGACGGAC    3120

TGCTAAATCG ACTGGTGGGT GTGGATAATC CCGACGAGGT GCTGCAGTTG CAATTGCTAA    3180

AGGAAAAGCA GACATCGAAT GGTATATTCA AGTGCTGGGA GCCGCACGAC AAAATACCGC    3240

CGGATACTCT AAGGAATCTA CTGGCCCGAT TCTTTGATCT GACCACTCCG CCATCGCGAC    3300

AGCTACTCAC CCTGCTGGCT GGATTCTGTG AGGACACCGC GGACAAGGAG CGGCTGGAGT    3360

TGCTGGTCAA CGATTCGTCG GCCTACGAGG ACTGGCGGCA CTGGCGGCTG CCGCACCTGC    3420

TGGACGTCCT CGAGGAGTTC CCTTCGTGCC GACCACCGGC TCCCCTTCTG CTTGCCCAAC    3480

TAACGCCGCT GCAGCCTCGC TTCTATTCCA TTTCCTCGTC GCCGCGCCGC GTTAGTGACG    3540

AAATCCACCT GACGGTGGCC ATCGTGAAGT ACCGTTGTGA AGATGGTCAG GGTGACGAGC    3600

GGTACGGCGT GTGCTCTAAC TATCTATCCG GCTTGCGGGC AGACGACGAG CTGTTCATGT    3660

TCGTGAGAAG CGCCTTGGGC TTCCATTTGC CCAGCGATCG GAGTCGTCCC ATTATTCTGA    3720

TTGGTCCTGG CACAGGAATA GCTCCATTCC GCTCCTTTTG GCAGGAGTTC CAGGTGCTAC    3780

GCGACCTTGA TCCCACGGCC AAATTGCCCA AGATGTGGCT CTTCTTTGGC TGCCGGAATC    3840

GGGATGTGGA CTTGTACGCC GAGGAGAAGG CAGAGCTACA GAAGGATCAA ATCCTAGACC    3900

GAGTTTTTCT CGCTCTGTCC AGGGAGCAGG CCATTCCGAA GACATATGTG CAGGACCTGA    3960

TTGAGCAGGA ATTCGATTCG TTGTACCAGT TGATTGTCCA GGAGCGGGGC CACATCTACG    4020

TCTGCGGCGA TGTCACAATG GCCGAGCATG TGTACCAGAC CATCAGGAAG TGCATTGCCG    4080

GCAAAGAGCA GAAAAGCGAG GCGGAAGTTG AGACATTTTT GCTAACACTG CGGGACGAAA    4140

GTCGCTACCA CGAGGACATC TTTGGCATCA CGCTGCGAAC GGCTGAGATA CACACAAAGT    4200

CAAGGGCCAC GGCCAGGATA CGAATGGCCT CCCAGCCCTA AGGATAGATA TTCGAAGTAA    4260

TCAAAATAGG AGGGTGACAT ATCCAAATTC GAGAGGAATA CCAAGCACTT GCTCTTTTTT    4320

TTCTTCCATA TTCAAATGCA ATTAAATATT GTCGCTTTGT TCATTACATA TTCGTATGAA    4380

TAACGTTTAA ATAAATTACA TTTTATTATT GATTCTAATG TACAAATCAA TTGTGAAATC    4440

AAAATCTAAA TGTTAAAATA TATTTCAAAT AAACGAATCG AAAAGGAATT C             4491
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-125-

CLAIMS

What is claimed is:

1. A method of regulating long term memory in an animal comprising inducing of expression of a dCREB2 gene or functional fragment thereof in the animal.

2. The method of Claim 1 wherein the dCREB2 gene encodes a cyclic 3',5'-adenosine monophosphate responsive activator isoform and inducing of said gene results in the potentiation of long term memory.

3. The method of Claim 2 wherein the activator isoform is dCREB2-a or an analogue thereof.

4. The method of Claim 2 wherein induction of the dCREB2 gene encoding a cyclic 3',5'-adenosine monophosphate responsive activator isoform activates the production of a protein which is necessary for the formation of long term memory.

5. The method of Claim 4 wherein the activator isoform is dCREB2-a or an analogue thereof.

6. The method Claim 1 wherein the dCREB2 gene encodes a repressor isoform and inducing of said gene results in the blocking of long term memory.

7. The method of Claim 6 wherein the repressor isoform is dCREB2-b or an analogue thereof.

-126-

8. A method of regulating long term memory in an animal
    comprising inducing repressor and activator isoforms
    of dCREB2 wherein long term memory is potentiated in
    the animal when the net amount of functional activator
    (ΔC) is greater than zero.

9. The method of Claim 8 wherein the repressor isoform is
    dCREB2-b or an analogue thereof and the activator
    isoform is dCREB2-a or an analogue thereof.

10. A method of identifying a substance capable of
    affecting long term memory in an animal comprising the
    determination that said substance alters the induction
    or activity of repressor and activator isoforms of
    dCREB2 from normal in the animal.

11. A method of enhancing long term memory formation in an
    animal comprising increasing the level of activator
    homodimer from normal in an animal.

12. The method of Claim 11 wherein the activator homodimer
    is a dCREB2a homodimer.

13. A method of enhancing long term memory formation in an
    animal comprising decreasing the level of activator-
    repressor heterodimer from normal in an animal.

14. The method of Claim 13 wherein the activator-repressor
    heterodimer is a dCREB2a-dCREB2b heterodimer.

15. A method of enhancing long term memory formation in an
    animal comprising decreasing the level of repressor
    homodimer from normal in an animal.

-127-

16. The method of Claim 15 wherein the repressor homodimer is a dCREB2b homodimer.

17. A method of identifying a substance capable of affecting long term memory in an animal comprising the determination that said substance alters from normal, in the animal, the formation of a dimer selected from the group consisting of: activator homodimer, activator-repressor heterodimer and repressor homodimer.

18. Isolated DNA encoding a cyclic 3',5'-adenosine monophosphate responsive transcriptional activator.

19. The isolated DNA of Claim 18 wherein the cyclic 3',5'-adenosine monophosphate responsive transcriptional activator is encoded by a *Drosophila* dCREB2 gene.

20. The isolated DNA of Claim 18 wherein the *Drosophila* dCREB2 gene codes for a dCREB2-a isoform.

21. The isolated DNA of Claim 18 which hybridizes to DNA having the sequence in Figure 1A (SEQ ID NO.: 1).

22. The isolated DNA of Claim 18 which encodes the amino acid sequence in Figure 1A (SEQ ID NO.: 2).

23. Isolated DNA encoding an antagonist of cyclic 3',5'-adenosine monophosphate-inducible transcription.

24. The isolated DNA of Claim 23 wherein the antagonist of cyclic 3',5'-adenosine monophosphate-inducible transcription is encoded by a *Drosophila* dCREB2 gene or a functional fragment thereof.

-128-

25.   The isolated DNA of Claim 24 wherein the *Drosophila*
      dCREB2 gene codes for a dCREB2-b isoform.

26.   An isolated DNA which encodes a *Drosophila* dCREB2 gene
      or a functional fragment thereof.

5   27.   The isolated DNA of Claim 26 wherein the *Drosophila*
          dCREB2 gene codes for an isoform selected from the
          group consisting of:
                a)   dCREB2-a;
                b)   dCREB2-b;
10   c)   dCREB2-c; and
                d)   dCREB2-d.

    28.   The isolated DNA of Claim 26 wherein the *Drosophila*
          dCREB2 gene codes for an isoform selected from the
          group consisting of:
15   a)   dCREB2-q;
                b)   dCREB2-r; and
                c)   dCREB2-s.

    29.   Isolated DNA encoding an enhancer-specific activator.

    30.   The isolated DNA of Claim 29 wherein the enhancer
20   specific activator is encoded by a *Drosophila* dCREB1
          gene or a functional fragment thereof.

    31.   The isolated DNA of Claim 30 which hybridizes to DNA
          having the sequence in Figure 5 (SEQ ID NO.: 7).

    32.   The isolated DNA of Claim 30 which encodes the amino
25   acid sequence in Figure 5 (SEQ ID NO.: 8).

    33.   Isolated DNA encoding a nitric oxide synthase of
          Drosophila (DNOS).

-129-

34.   The DNA of Claim 33 encoding a DNOS of neuronal locus.

35.   The DNA of Claim 33 encoding a DNOS which contains putative heme, calmodulin, FMN, FAD and NADPH binding site domains.

5   36.   A method for assessing the effect of a drug on long term memory formation comprising:
    a)   administering said drug to Drosophila;
    b)   subjecting the Drosophila to classical conditioning and to at least one odorant and
10   electrical shock; and
    c)   assessing the performance index of said classical conditioning,
    wherein the effect of said drug occurs when said drug alters said performance index from normal.

15 37.   A method of Claim 36 wherein said drug affects long term memory formation by altering the induction or activity of repressor and activator isoforms of dCREB2.

FIGURE 1A

```
  1    ATGGACAACAGCATCGTCGAGGAGAACGGCAACTCGTCGGCGGCATCGGGCTCCAATGAC
  1    M  D  N  S  I  V  E  E  N  G  N  S  S  A  A  S  G  S  N  D

 61    GTGGTCGATGTCGTTGCCCAACAGGCGGCGGCAGCGGTGGGCGGCGGCGGTGGAGGAGGA
 21    V  V  D  V  V  A  Q  Q  A  A  A  A  V  G  G  G  G  G  G  G

121    GGAGGCGGCGGCGGTGGTGGTAACCCCCAGCAGCAGCAACAGAACCCACAAAGTACAACG
 41    G  G  G  G  G  G  G  N  P  Q  Q  Q  Q  Q  N  P  Q  S  T  T

181    GCCGGCGGTCCAACGGGTGCGACGAACAACGCCCAGGGGAGGCGGAGTGTCCTCCGTGCTG
 61    A  G  G  P  T  G  A  T  N  N  A  Q  G  G  G  V  S  S  V  L

241    ACCACCACCGCCAACTGCAACATACAATACCCCATCCAGACGCTGGCGCAGCACGGACTG
 81    T  T  T  A  N  C  N  I  Q  Y  P  I  Q  T  L  A  Q  H  G  L

301    CAGGTGAGCATTTGGGGACCGGGTGCTTGGTGTCAACTGTCGAGTGTCAGGTGTTACGGA
101    Q  V  S  I  W  G  P  G  A  W  C  Q  L  S  S  V  R  C  V  G
                 Exon 2

361    TCCCAGCCAGAAGTGGCTACCAAGGATGTGCAGTCCGTGATACAGGCCAATCCCTCGGGA
121    S  Q  P  E  V  A  T  K  D  V  Q  S  V  I  Q  A  N  P  S  G

421    GTCATACAGACAGCAGCTGGAACCCAGCAGCAGCAACAGGCGCTGGCCGCCGCCACAGCG
141    V  I  Q  T  A  A  G  T  Q  Q  Q  Q  Q  A  L  A  A  A  T  A

481    ATGCAGAAGGTGGTCTACGTGGCCAAGCCGCCGAACTCGACGGTCATCCACACGACGCCT
161    M  Q  K  V  V  Y  V  A  K  P  P  N  S  T  V  I  H  T  T  P

541    GGCAATGCAGTGCAAGTGCGTAACAAAATCCCTCCAACCTTTCCATGTAAGATCAAGCCC
181    G  N  A  V  Q  V  R  N  K  I  P  P  T  F  P  C  K  I  K  P
                 Exon 4

601    GAACCGAACACGCAGCACCCGGAGGACAGCGACGAGAGTCTGTCGGACGACGATTCCCAG
201    E  P  N  T  Q  H  P  E  D  S  D  E  S  L  S  D  D  D  S  Q

661    CACCACCGCAGCGAGCTGACGCGACGGCCGTCGTACAATAAGATCTTCACCGAGATCAGC
221    H  H  R  S  E  L  T  R  R  P  S  V  N  K  I  F  T  E  I  S
                       P-box

721    GGTCCGGACATGAGCGGCGCATCGCTTCCCATGTCCGACGGCGTGCTCAATTCCCAGCTG
241    G  P  D  M  S  G  A  S  L  P  M  S  D  G  V  L  N  S  Q  L
                 Exon 6

781    GTGGGGACCGGAGCGGGGGGCAATGCGGCGAACAGCTCCCTGATGCAATTGGATCCCACG
261    V  G  T  G  A  G  G  N  A  A  N  S  S  L  M  Q  L  D  P  T

841    TACTACCTGTCCAATCGGATGTCCTACAACACCAACAACAGCGGGATAGCCGAGGATCAG
281    Y  Y  L  S  N  R  M  S  Y  N  T  N  N  S  G  I  A  E  D  Q

901    ACCCGTAAGCGCGAGATCCGGCTGCAGAAGAACAGGGAGGCGGCGCGTGAGTGCCGGCGC
301    T  R  K  R  E  I  R  L  Q  K  N  R  E  A  A  R  E  C  R  R
       Basic region ——→

961    AAGAAGAAGGAGTACATCAAGTGCCTGGAGAATCGAGTGGCGGTGCTAGAGAACCAAAAC
321    K  K  K  E  V  I  K  C  L  E  N  R  V  A  V  L  E  N  Q  N
                                      Leucine zipper ——→

1021   AAAGCGCTCATCGAGGAGCTGAAGTCGCTCAAGGAGCTCTATTGTCAGACCAAGAACGAT
341    K  A  L  I  E  E  L  K  S  L  K  E  L  Y  C  Q  T  K  N  D

1081   TGA
361    END
```

FIGURE 1B

```
dCREB2   RKRED L L D KNREAAR E C R K K K E V D K C L E N R V A V L E N Q N E L I E E L K S L K E L Y C
CREB     RKREVRL NKNREAAR E C R K K K E Y V K C L E N R V A V L E N Q N K T L I E E L K A L K D L Y C
CREM I   RKRELRL NKNREAAR E C R K K K E Y V K C L E N R V A V L E N Q N K T L I E E L K A L K D L Y C
ATF-1    LKREIRL NKNREAAR E C R K K K E Y V K C L E N R V A V L E N Q N K T L I E E L K T L K D L Y S
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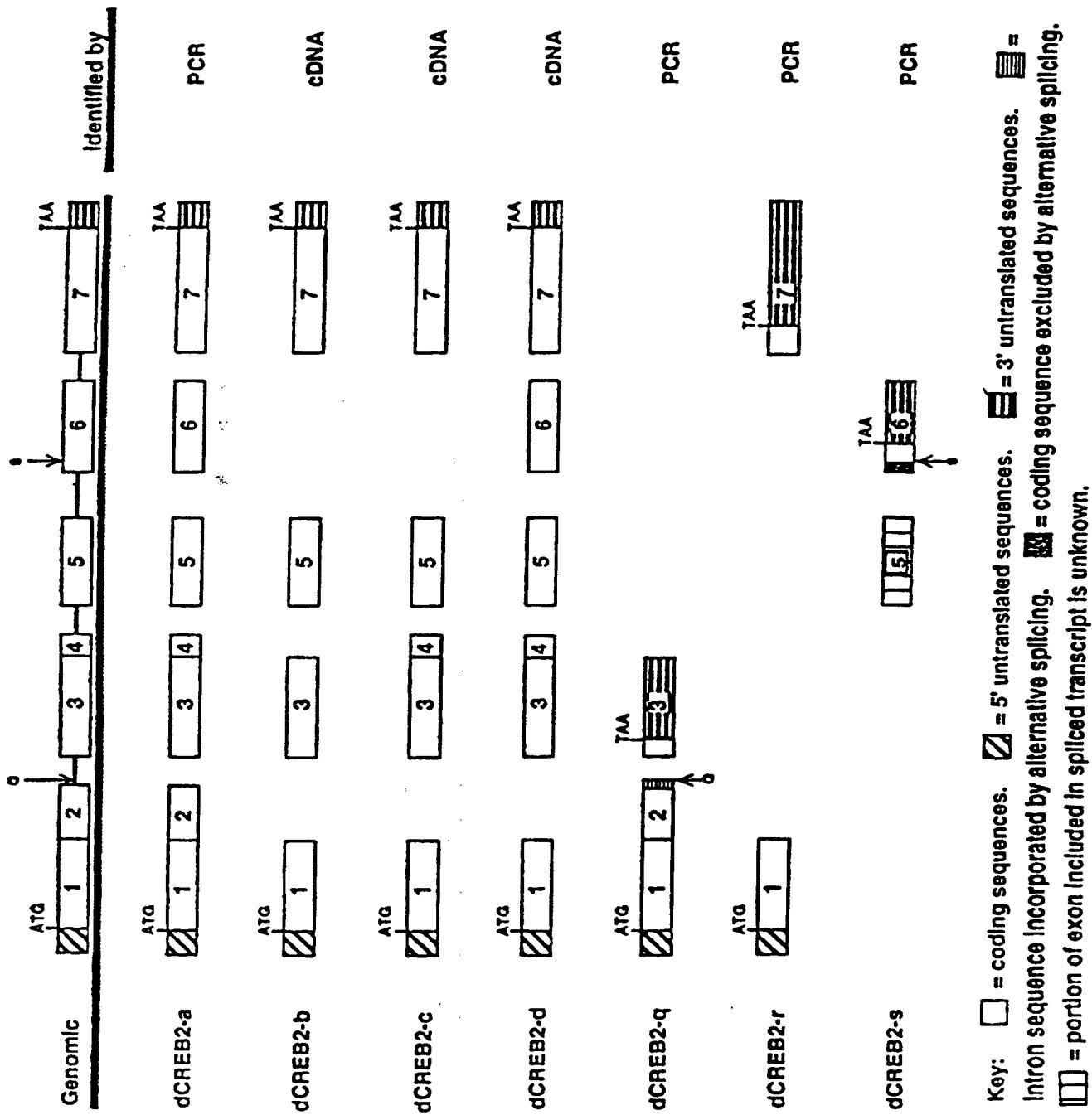
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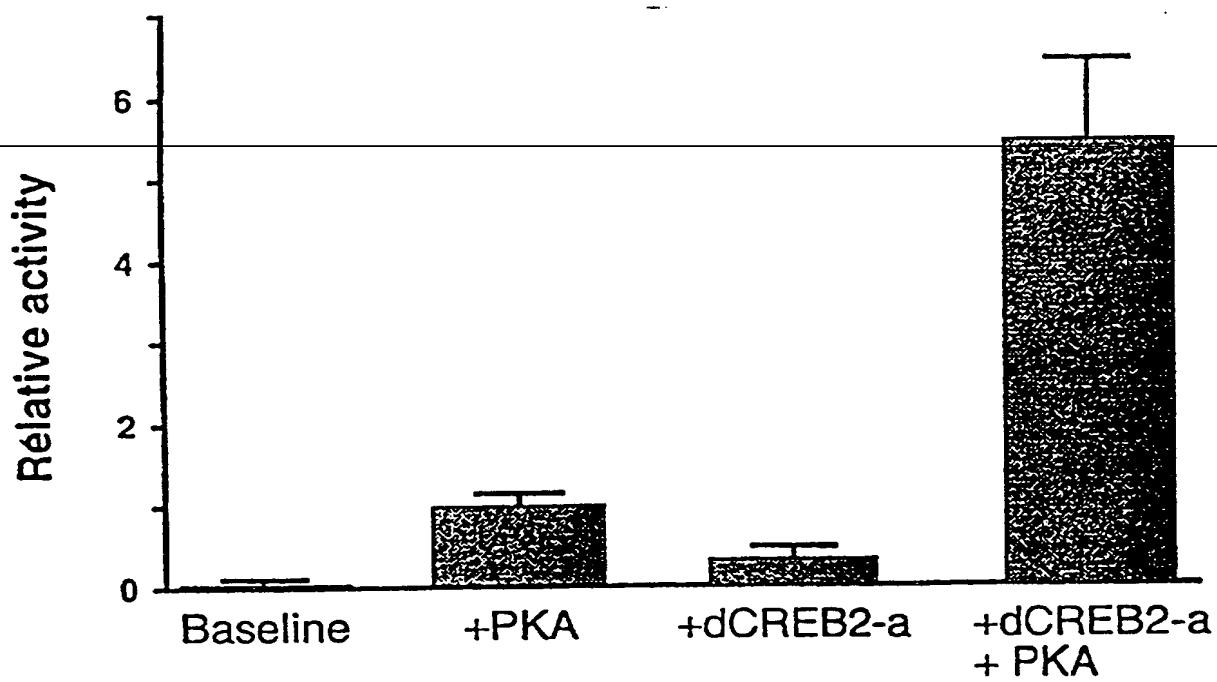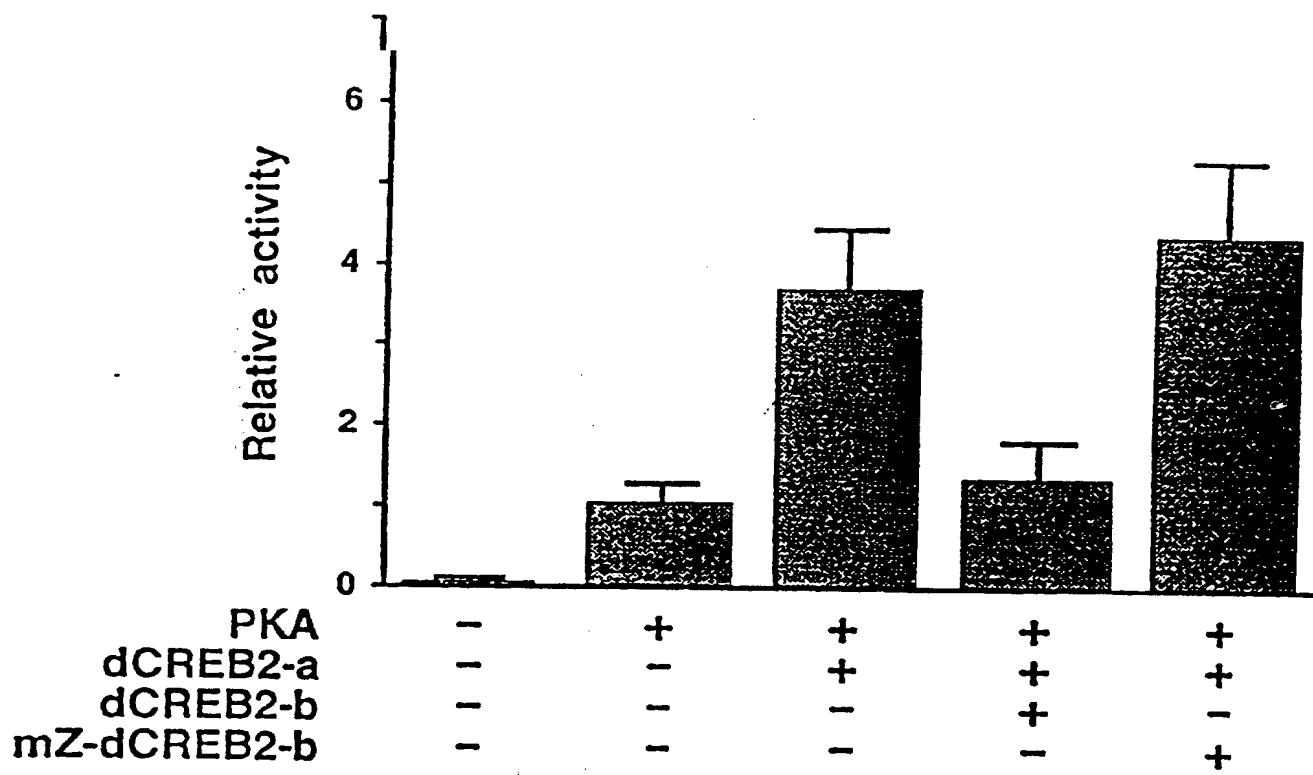
Figure 3

Figure 4

```
  1   ATGTTACTCGGAGAAATATGTTTTCTACTTTCACATCGTTAGATGCTGCTACCGCTACA
  1    M  L  L  G  E  M  M  F  S  T  F  T  S  L  D  A  A  T  A  T

 61   ACCAACACCGGTGAATTCTTAATGAATGAATCTCCAAGGCAAGAAGCCGGTGACTTAATG
 21    T  M  T  G  E  F  L  M  M  E  S  <P>  R  Q  E  A  G  D  L  M

121   TTGGATAGTCTGGATTTCAACATTATGGGCGAAAACCTGGCAGATGATTTCCAGACCTCG
 41    L  D  S  L  D  F  M  I  M  G  E  M  L  A  D  D  F  Q  T  S

181   GCTTCACCAGCTTCGGAGGACAAGATGACTCCTTTCGTTGTTGATACCAATGTTTTTGAA
 61    A  S  P  A  S  E  D  K  M  T  <P>  F  V  V  D  T  M  V  F  E

241   TCCGTCTTCAAGAACACCGAAGATACCCTTCTAGGAGATATCGACAATGTTGGTATTGTT
 81    S  V  F  K  M  T  E  D  T  L  L  G  D  I  D  M  V  G  I  V

301   GACACGGAGTTGAAGGAGATGTTCGATTTGGTTGACTCGGAAATCAATAACGGCACTCCT
101    D  T  E  L  K  E  M  F  D  L  V  D  S  E  I  M  M  G  T  <P>

361   ATCAAGCAGGAAGAAAAGGATGATTTGGAATTTACTTCAAGATCCCAGTCCACCTCAGCT
121    I  K  Q  E  E  K  D  D  L  E  F  T  S  R  S  Q  S  T  S  A

421   CTCTTGTCGTCGAAATCGACTTCTGCTTCTCCAGCTGATGCTGCCGCTGCATGTGCAAGT
141    L  L  S  S  K  S  T  S  A  S  <P>  A  D  A  A  A  A  C  A  S

481   CCTTCGTCATCGTCTTGTAAAAGATCCTATTCTTCTGCTCAGCTAGAAACTACGGGTTCG
161    P  S  S  S  S  C  K  R  S  Y  S  S  A  Q  L  E  T  T  G  S

541   GATGCTCCAAAGAAAGATAAGCTGGGCTGCACCCCTTACACTAGAAAACAGAGAAACAAT
181    D  A  P  K  K  D  K  L  G  C  T  P  V  T  R  K  Q  R  M  M

601   CCATTACCTCCGGTCATTCCAAAGGGTCAGGATGTTGCTTCTATGAAAAGGGCAAGAAAC
201    P  L  P  P  V  I  P  K  G  Q  D  V  A  S  M  (K)(R) A (R) M
                                                   ━━━━━━━━━━━━━━
                                                   Basic region→

661   ACTGAGGCCGCAAGAAGATCAAGAGCCAGAAAAATGGAAAGAATGTCCCAACTTGAAGAA
221    T  E  A  A (R)(R) S (R) A (R)(K) M  E (R) M  S  Q  [L] E  E
      ━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━ ▓▓▓▓▓▓
                                                         Leucine

721   AAGTGTCAAAGCTTGTTGAAGGAAAACGACGACTTGAAAGCTCAAGTTCAAGCTTTGAAG
241    K  C  Q  S  [L] L  K  E  M  D  D  [L] K  A  Q  V  Q  A  [L] K
      ▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓▓
      zipper——→

781   AAATTACTTGGACAACAA
261    K  L  L  G  Q  Q
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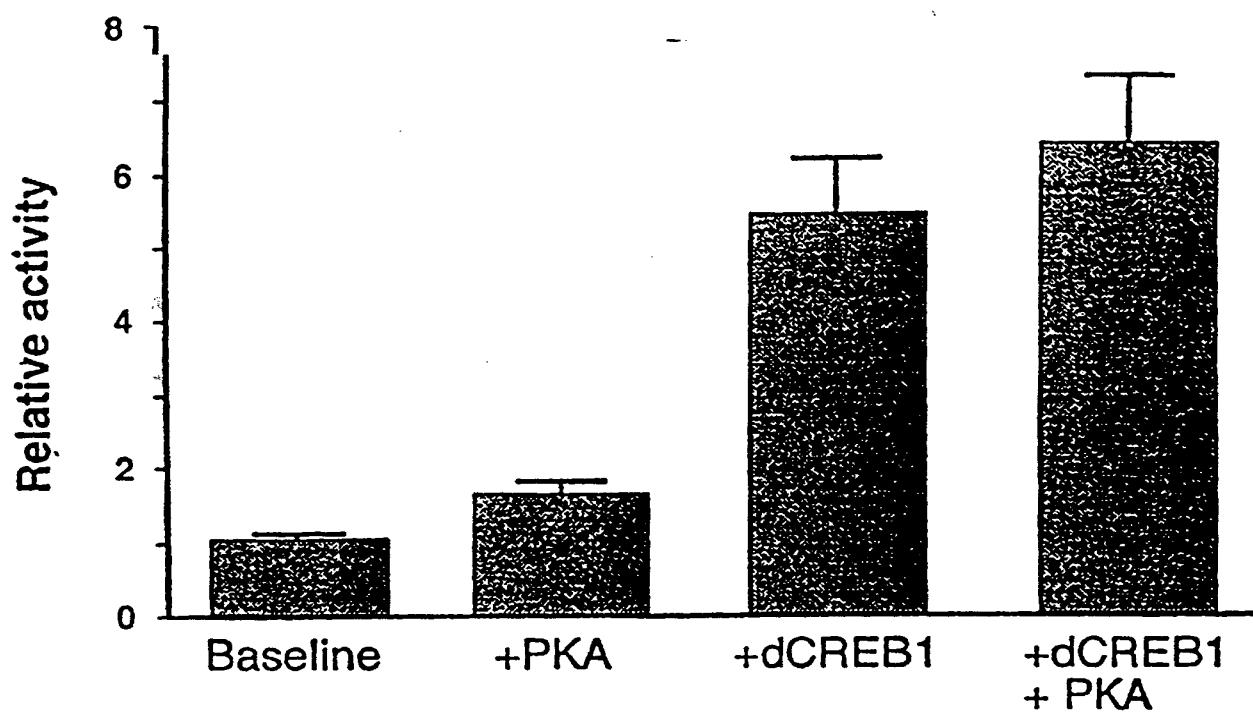
Figure 5

Figure 6

Figure 7A

LANE #	1	2	3	4	5	6
HEAT SHOCK	-	-	+	+	+3	+3
FLIES	wt	CREB	wt	CREB	wt	CREB

— CREB TRANSGENE RNA

Figure 7B

LANE #	1	2	3	4	5	6	7	8	9	10	11	12
HEAT SHOCK	-	-	+	+	+1	+1	+3	+3	+9	+9	+24	+24
FLIES	wt	CREB	wt	CREB	wt	CREB	wt	CREB	wt	CREB	wt	CREB

— dCREB2B PROTEIN

Figure 7C

lane	1	2	3	4	5	6	7	8
blocker	wt	m	wt	m	wt	m	wt	m
hs	-	-	+	+	+3	+3	+6	+6
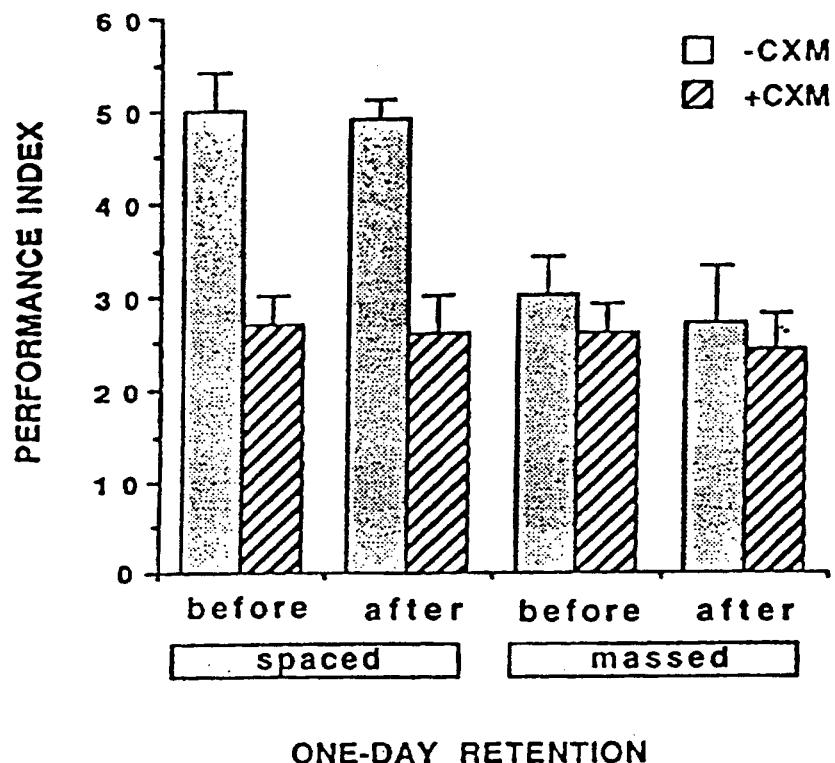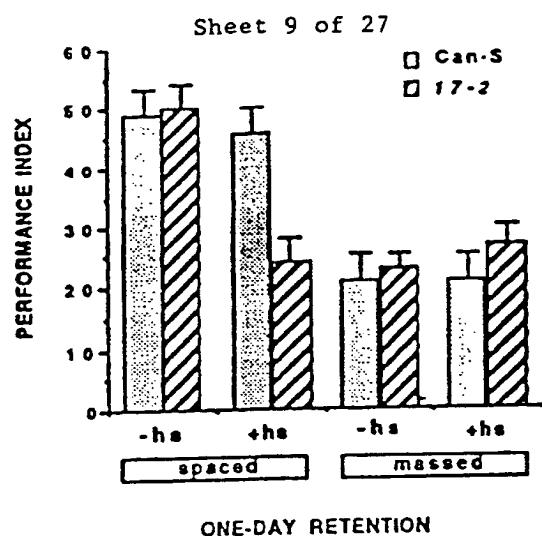
ONE-DAY RETENTION

Figure 8

Figure 9A

Figure 9B
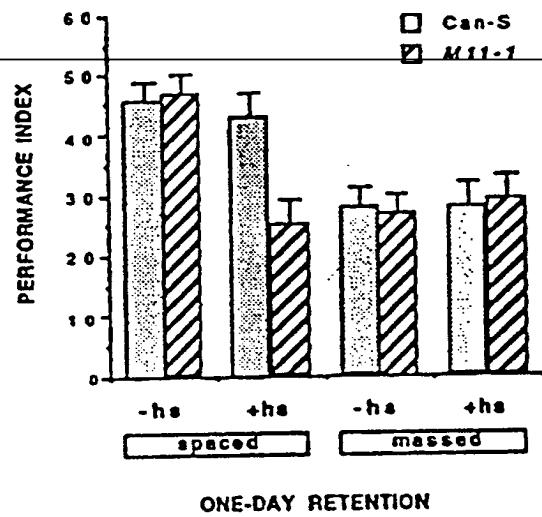
Figure 9C
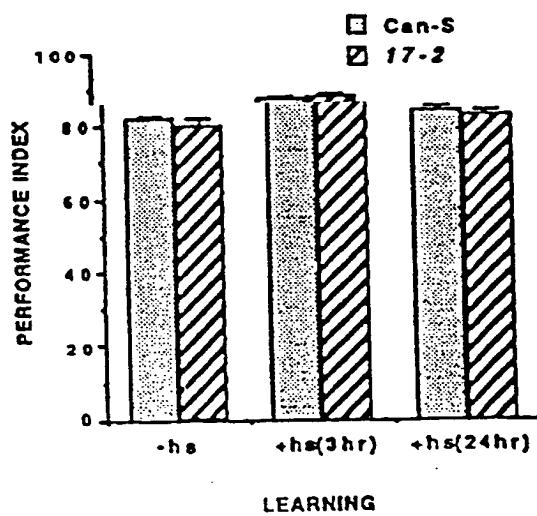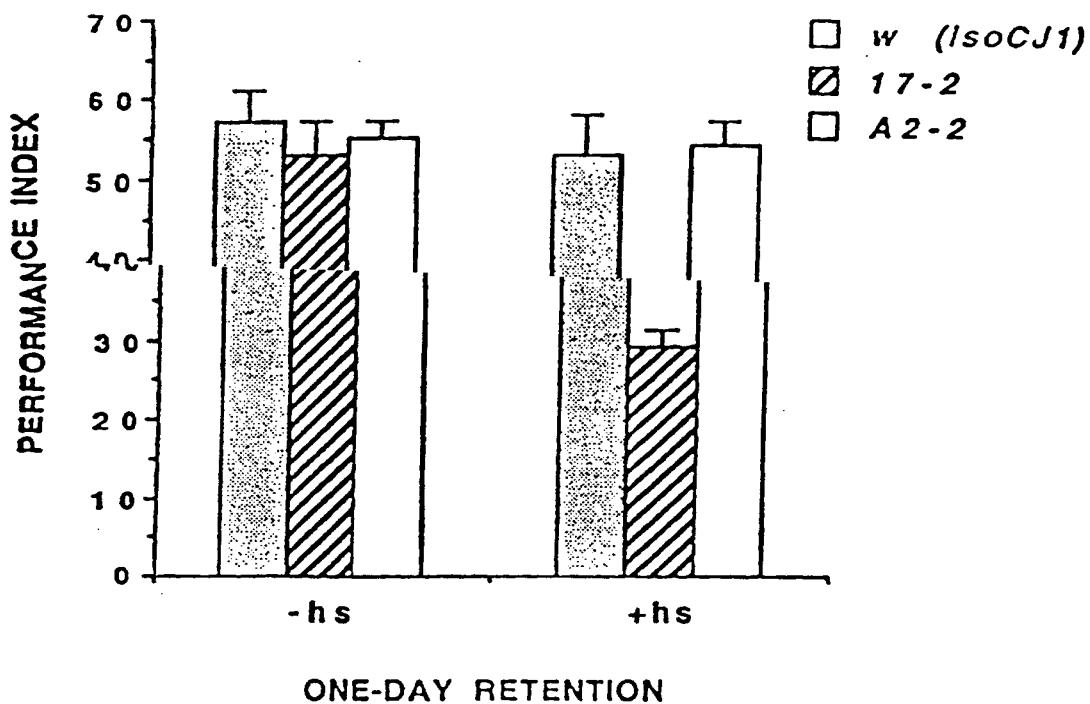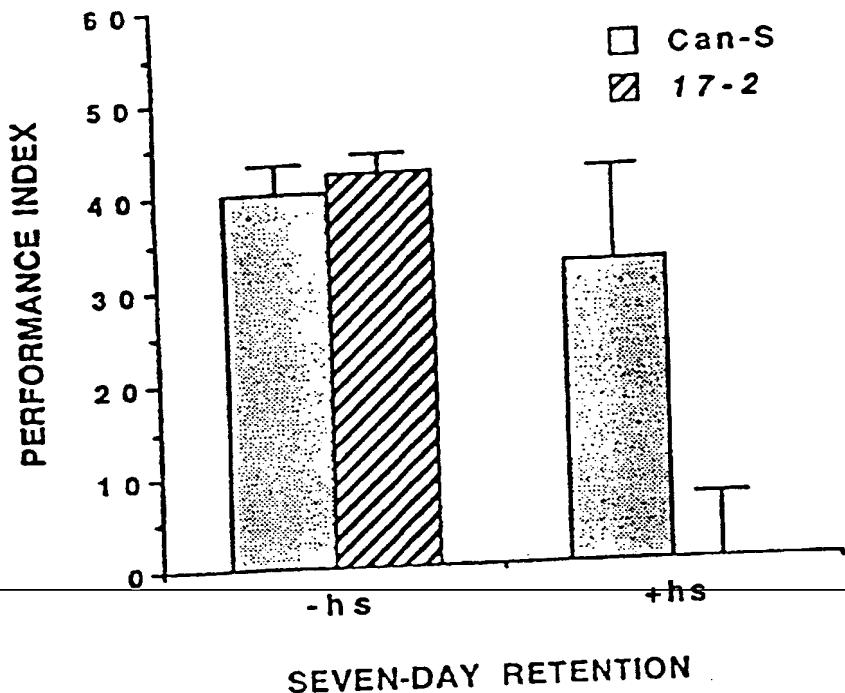
Figure 10

Figure 11

ONE-DAY RETENTION
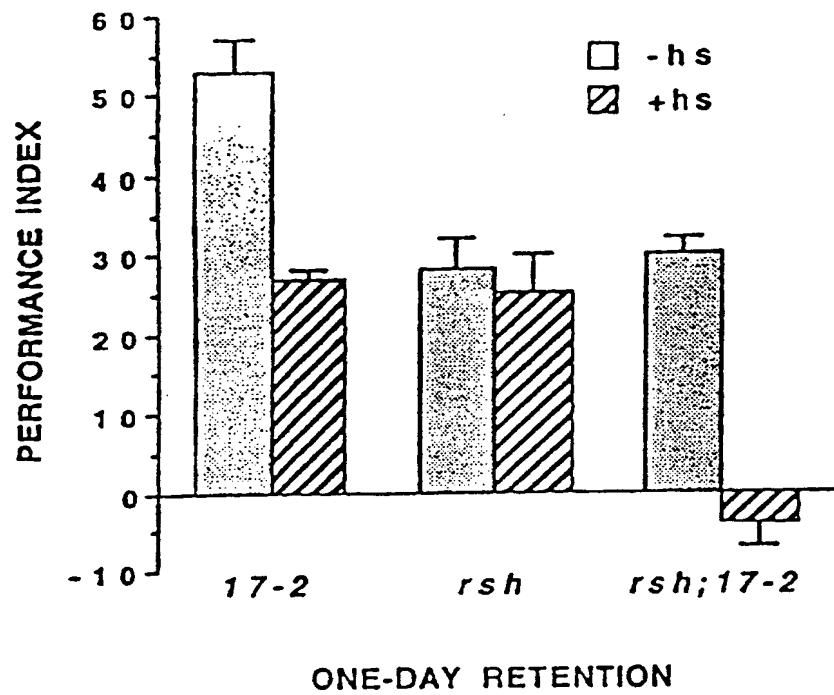
Figure 12
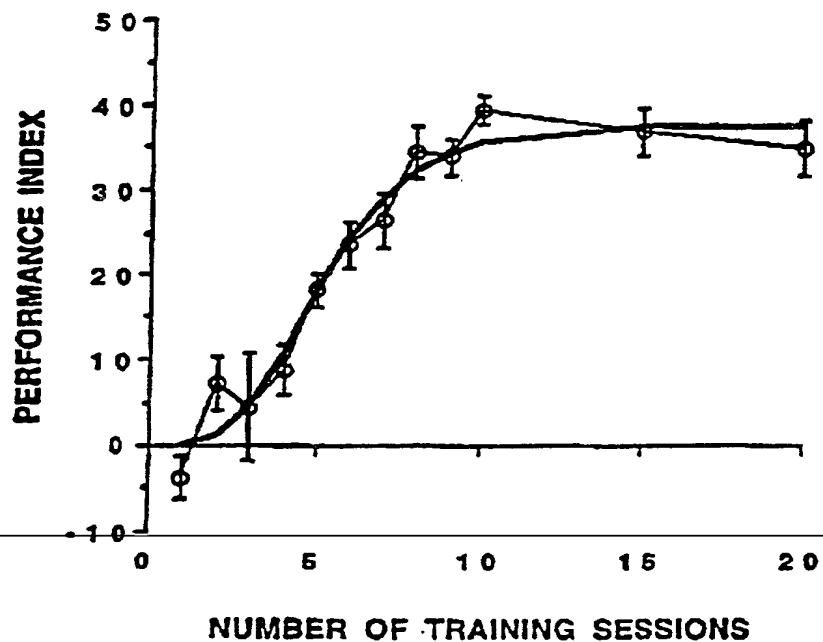
Figure 13A

Figure 13B
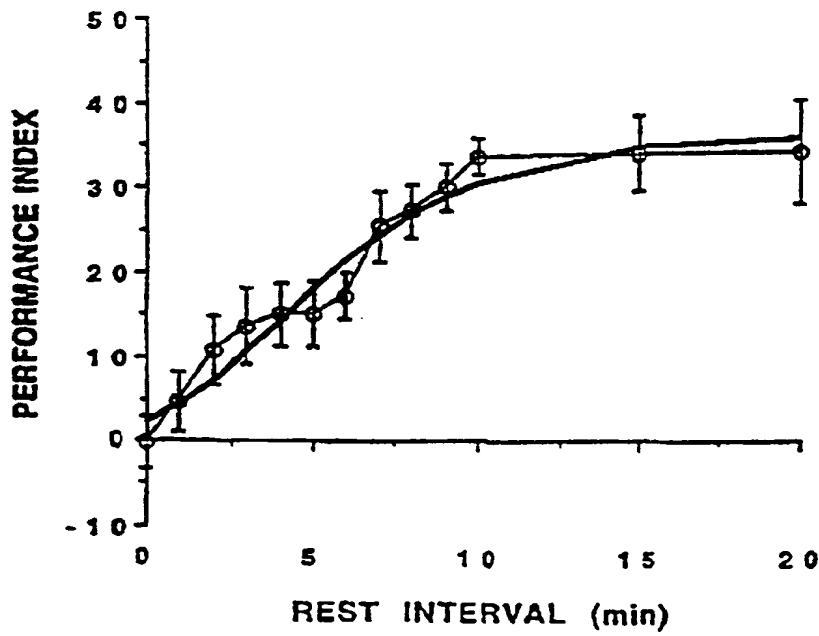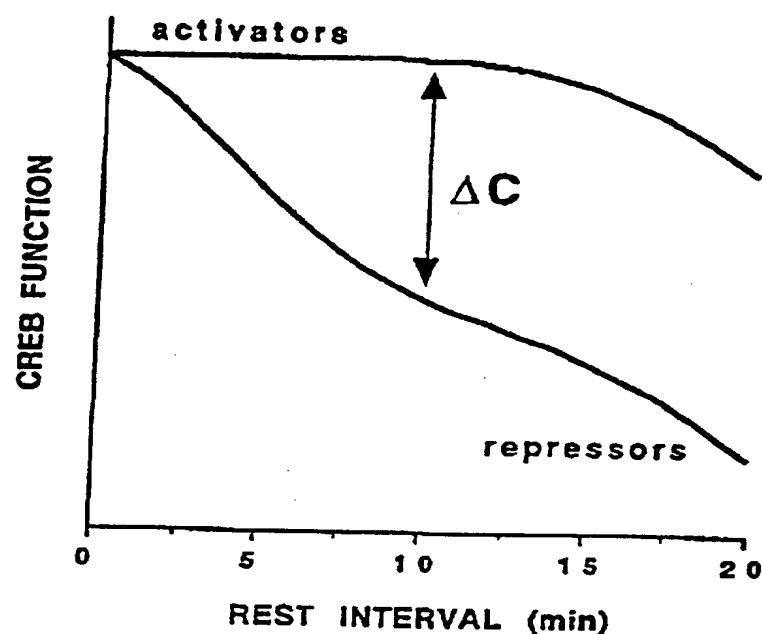
Figure 14

Figure 15A

7-DAY RETENTION

Figure 15B

7-DAY RETENTION
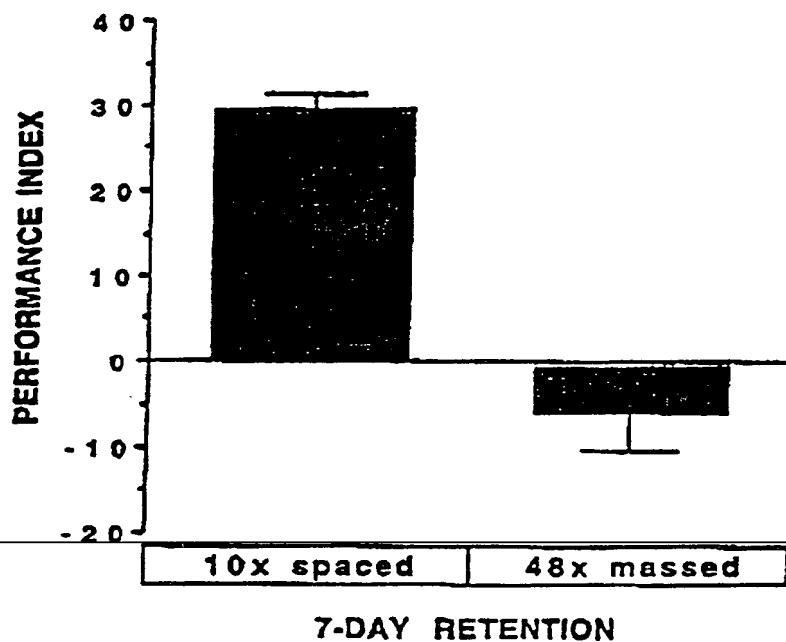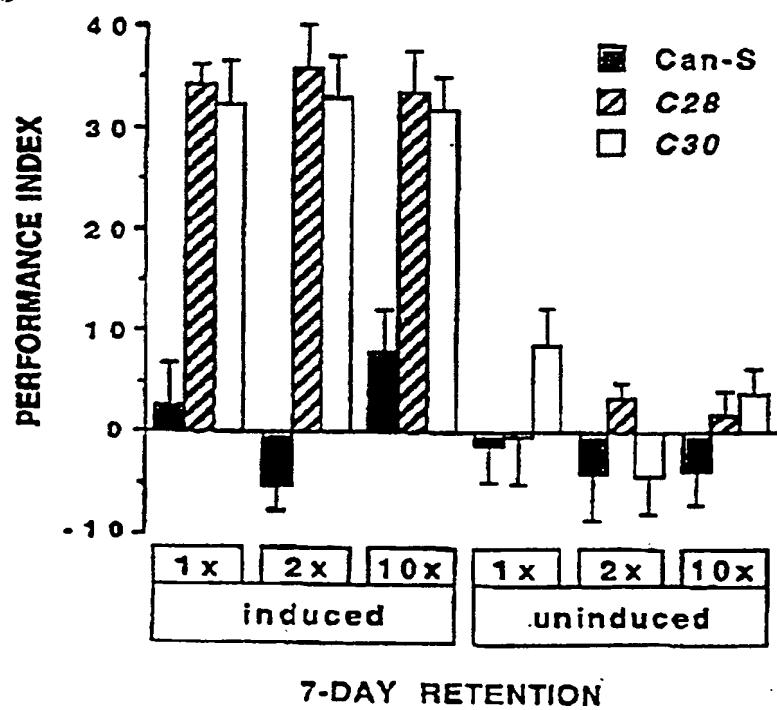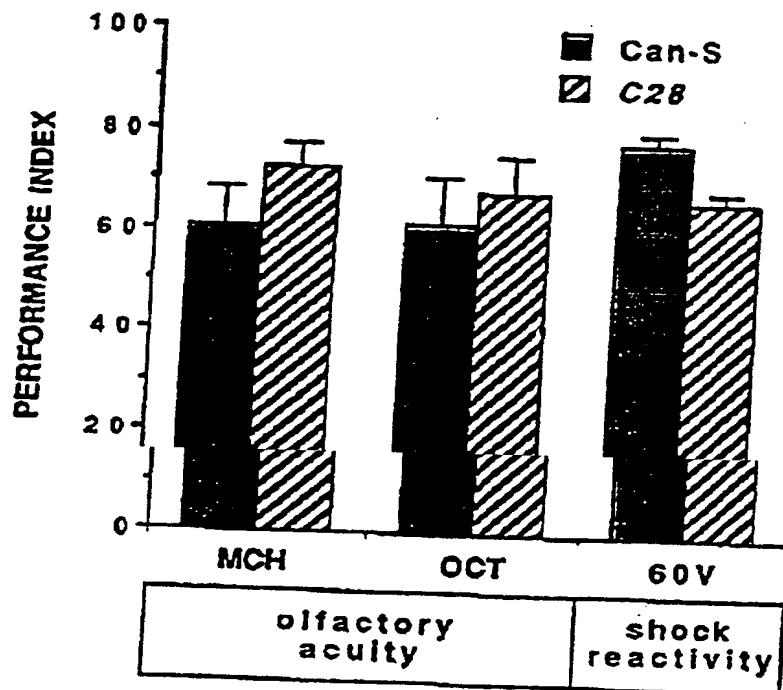
Figure 15C

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DNOS     MSQ------H FTSIFENLRF VTIKRATNAQ QQQQQQQQQQ L---------     35
BENOS    MGN------- ---------L --KSVG---- --QE--PGPP ----------     14
RNNOS    MEENTFGVQQ IQPNVISVRL FKRKVGGLGF LVKERVSKPP VIISDLIRGG     50
MMNOS    MAC------- -----PWKFL FKVKSY---- --QSDLKEEK ----------     22


DNOS     QQQQQQLQQQ ---------- -----KAQTQ QQNSRKIKTQ ATPTLNGNGL     70
BENOS    ----CGL--- ---------- ---------G L----GLG-- ----LGLCGK     28
RNNOS    AAEQSGLIQA GDIILAVNDR PLVDLSYDSA LEVLRGIASE THVVLILRGP    100
MMNOS    -----DI--- ---------- ---------N N----NVK-- ----KTPCAV     35


DNOS     LS-GNPNGGG GDSSPSHEVD HPGGAQGAQ- ---------- -------AAG    101
BENOS    QG-------- -PASPAPEPS RAPAPATP-- ---------- ----------     47
RNNOS    EGFTTHLETT FTGDGTPKTI RVTQPLGPPT KAVDLSHQPS ASKDQSLAVD    150
MMNOS    LS-------- PTIQDDPKSH QNGSPQLL-- ---------- --------- --     55


DNOS     GLPSLSGTPL RHH------- ---KRASIST ASPPIRERRG ----------    131
BENOS    ------HAPD HS-------- ---------- -PAPNS---- ----------     58
RNNOS    RVTGLGNGPQ HAQGHGQGAG SVSQANGVAI DPTMKSTKAN LQDIGEHDEL    200
MMNOS    ------TGTA QN-------- ---------- --VPESL--- ----------     66


DNOS     --TNTSIVVE LDGSGSGSGS GG-------- GGVGVGQGAG CPPSGSCTAS    171
BENOS    -----PTLT- ---------- ---------- ------R--- ------PPEG     67
RNNOS    LKEIEPVLSI LNSGSKATNR GGPAKAEMKD TGIQVDRDLD GKSHKAPPLG    250
MMNOS    -----DKLHV ---------- ---------- ---------- ------TSTR     75


DNOS     GKSSRELSPS PKNQQQPRXM SQDYRSR--- -AGSFMHLDD EGRSLLMRKP    217
BENOS    ---------- ---------- ---------- ---------- -------PKF     70
RNNOS    GDNDRVFNDL WGKDNVPVIL NNPYSEKEQS PTSGKQSPTK NGSPSRCPRF    300
MMNOS    ---------- ---------- ---------- ---------- -------PQY     78

                                                   / /
DNOS     MRLKNIEGRP EVYDTLHCKG REILSCSKAT CTSSIMN--- -IGNAAVEAR    263
BENOS    PRVKNWELGS ITYDTLCAQS QQDGPCTPRR CLGSLVLPRK LQTRPSPGPP    120
RNNOS    LKVKNWETDV VLTDTLHLKS TLETGCTEHI CMGSIMLPSQ -HTRKPEDVR    349
MMNOS    VRIKNWGSGE ILHDTLHHKA TSDFTCKSKS CLGSIMNPKS LTRGPRDKPT    128


DNOS     KSDLILEHAK DFLEQYFTSI KRTSCTAHET RWKQVRQSIE TTGHYQLTET    313
BENOS    PAEQLLSQAR DFINQYYSSI KRSGSQAHEE RLQEVEAEVA STGTIHLRES    170
RNNOS    TKDQLFPLAK EFLDQYYSSI KRFGSKAHMD RLEEVNKEIE STSTYQLKDT    399
MMNOS    PLEELLPHAI EFINQYYGSF KEAKIEEHLA RLEAVTKEIE TTGTYQLTLD    178

                  ------Heme------
DNOS     ELIYGAXLAW RNSSRCIGRI QWSKLQVFDC RYVTTTSGMF EAICNHIKYA    363
BENOS    ELVFGAKQAW RNAPRCVGRI QWGKLQVFDA RDCSSAQEMF TYICNHIKYA    220
RNNOS    ELIYGAXHAW RNASRCVGRI QWSKLQVFDA RDCTTAHGMF NYICNHVKYA    449
MMNOS    ELIFATKMAW RNAPRCIGRI QWSNLQVFDA RNCSTAQEMF QHICRHILYA    228


DNOS     TNKGNLRSAI TIFPQRTDAK HDYRIWNNQL ISYAGYKQAD GKIIGDPMNV    413
BENOS    TNRGNLRSAI TVFPQRAPGR GDFRIWNSQL VRYAGYRQQD GSVRGDPANV    270
RNNOS    TNKGNLRSAI TIFPQRTDGK HDFRVWNSQL IRYAGYKQPD GSTLGDPANV    499
MMNOS    TNNGNIRSAI TVFPQRSDGK HDFRLWNSQL IRYAGYQMPD GTIRGDAATL    278


DNOS     EFTEVCTKLG WKSKGSEWDI LPLVVSANGH DPDYFDYPPE LILEVPLTHP    463
BENOS    EITELCIQHG WTPGNGRFDV LPLLLQAPDE APELFVLPPE LVLEVPLGAP    320
RNNOS    QFTEICIQQG WKAPRGRFDV LPLLLQANGN DPELFQIPPE LVLEVPIRHP    549
MMNOS    EFTQLCIDLG WKPRYGRFDV LPLVLQADGQ DPEVFEIPPD LVLEVTMEHP    328


DNOS     KFEWFSDLGL RWYALPAVSS MLFDVGGIQF TATTFSGWYM STEIGSRNLC    513
BENOS    HTGVVRGPGL RWYALPAVSN MLLEIGGLEF SAAPFSGWYM STEIGTRNLC    370
RNNOS    KFDWFKDLGL KWYGLPAVSN MLLEIGGLEF SACPFSGWYM GTEIGVRDYC    599
MMNOS    KYEWFQELGL RWYALPAVAN MLLEVGGLEF PACPFNGWYM GTEIGVRDFC    378
```

Figure 16A

```
DNOS    DTNRRNMLET VALKMQLDTR TPTSLWKDKA VVEMNIAVLH SYQSRNVTIV    563
BENOS   PPHRYNILED VAVCMDLDTR TTSSLWKDKA AVEINLAVLH SFQLAKVTIV    420
RNNOS   DNSRYNILEE VAKKMDLDMR KTSSLWKDQA LVEINIAVLY SFQSDKVTIV    649
MMNOS   DTQRYNILEE VGRRMGLETH TLASLWKDRA VTEINVAVLH SFQKQNVTIH    428


DNOS    DHHTASESFM KHFENESKLR NGCPADWIWI VPPLSGSITP VFHQEMALYY    613
BENOS   DHHAATVSFM KHLDNEQKAR GGCPADWAWI VPPIYGSLPP VFHQEMVNYI    470
RNNOS   DHHSATESFI KHMENEYRCR GGCPADWVWI VPPMSGSITP VFHQEMLNYR    699
MMNOS   DHHTASESFM KHMQNEYRAR GGCPADWIWL VPPVSGSITP VFHQEMLNYV    478


                        ————————CaM————————
DNOS    LKPSFEYQDP AWRTHVWKKG RGESKGKKPR RKFNFKQIAR AVKFTSKLFG    663
BENOS   LSPAFRYQPD PWKG---SAT KGAG---ITR KK-TFKEVAN AVKISASLMG    513
RNNOS   LTPSFEYQPD PWNTHVWKGT NGTP---TKR RAIGFKKLAE AVKFSAKLMG    746
MMNOS   LSPFYYYQIE PWKTHIWQNE KLRP----RR REIRFRVLVK VVFFASMLMR    524


DNOS    RALSKRIKAT VLYATETGKS EQYAKQLCEL LGHAFNAQIY CMSDYDISSI    713
BENOS   TLMAKRVKAT ILYASETGRA QSYAQQLGRL FRKAFDPRVL CMDEYDVVSL    563
RNNOS   QAMAKRVKAT ILYATETGKS QAYAKTLCEI FKHAFDAKAM SMEEYDIVHL    796
MMNOS   KVMASRVRAT VLFATETGKS EALARDLATL FSYAFNTKVV CMDQYKASTL    574


DNOS    EHEALLIVVA STFGNGDPPE NGELFSQELY AMRVQESSEH GLQDSSIGSS    763
BENOS   EHEALVLVVT STFGNGDPPE NGESFAAALM EMSGPYNS-- ---SPRPEQH    608
RNNOS   EHEALVLVVT STFGNGDPPE NGEKFGCALM EMRHP----- ---NSVQEER    838
MMNOS   EEEQLLLVVT STFGNGDCPS NGQTLKKSL- ---------- ----------    603


DNOS    KSFMKASSRQ EFMKLPLQQV KRIDRWDSLR GSTSDTFTEE TFGPLSNVRF    813
BENOS   KSYK---IR- -FNSVS-CSD PLVSSWRRKR KESSNT---D SAGALGTLRF    649
RNNOS   KSYK---VR- -FNSVS-SYS DSRKSSGDGP DLRDNF---E STGPLANVRF    879
MMNOS   --FM---LR- ---------- ---------- ELNH-^^--- ------TFRY    615


                        ————————FMN————————
DNOS    AVFALGSSAY PNFCAFGQYV DNILGELGGE RLLRVAYGDE MCGQEQSFRK    863
BENOS   CVFGLGSRAY PHFCAFARAV DTRLEELGGE RLLQLGQGDE LCGQEEAFRG    699
RNNOS   SVFGLGSRAY PHFCAFGHAV DTLLEELGGE RILKMREGDE LCGQEEAFRT    929
MMNOS   AVFGLGSSMY PQFCAFAHDI DQKLSHLGAS QLAPTGEGDE LSGQEDAFRS    665


DNOS    WAPEVFKLAC ETFCLDPEES --LSDASLAL QNDSLTVNTV RLVPSANKGS    911
BENOS   WAKAAFQASC ETFCVGEEAK --AAAQDIFS PKRSWKRQRY RLSAQAEGLQ    747
RNNOS   WAKKVFKAAC DVFCVGDDVN IEKPNNSLIS NDRSWKRNKF RLTYVAEAPD    979
MMNOS   WAVQTFRAAC ETFDVRSKHH --IQIPKRFT SNATWEPQQY RLIQSPEPLD    713


                                                  ————
DNOS    LDSSLSKYHN KKVHCCKAKA KPH-NLTRLS EGAKTTMLLE ICAPGLEYEP    960
BENOS   LLPGLIHVHR RKMFQATVLS VENLQSSKST RATILVRLDT AGQEGLQYQP    797
RNNOS   LTQGLSNVHK KRVSAARLLS RQNLQSPKFS RSTIFVRLHT NGNQELQYQP    1029
MMNOS   LNRALSSIHA KNVFTMRLKS QQNLQSEKSS RTTLLVQLTF EGSRGPSYLP    763


        -FAD·PPi——
DNOS    GDHVGIFPAN RTELVDGLLN RLVGVDNPDE VLQLQLLKEK QTSNGIFKCW    1010
BENOS   GDHIGISAPN RPGLVEALLS RVEDPPPPTE SVAVEQL-EK GSPGGPPPSW    846
RNNOS   GDHLGVFPGN HEDLVNALIE RLEDAPPANH VVKVEMLEER NTALGVISNW    1079
MMNOS   GEHLGIFPGN QTALVQGILE RVVDCPTPHQ TVCLEVLDES G------SYW    807


DNOS    EPHDKIPPDT LRNLLARFFD LTTPPSRQLL TLLAGFCEDT ADKERLELLV    1060
BENOS   VRDPRLPPCT VRQALTFFLD ITSPPSPRLL RLLSTLAEEP SEQQELETLS    896
RNNOS   KDESRLPPCT IFQAFKYYLD ITTPPTPLQL QQFASLATNE KEKQRLLVLS    1129
MMNOS   VKDKRLPPCS LSQALTYFLD ITTPPTQLQL HXLARFATDE TDRQRLEALQ    857

                                                  —FAD·ISO—
DNOS    NDSSAYEDWR HWRLPHLLDV LEEFPSCRPP APLLLAQLTP LQPRFYSISS    1110
BENOS   QDPRRYEEWK LVRCPTLLEV LEQFPSVALP APLLLTQLPL LQPRYYSVSS    946
RNNOS   KGLQEYEEWK WGKNPTMVEV LEEFPSIQHP ATLLLTQLSL LQPRYYSISS    1179
MMNOS   Q-RSIXNDWK FSHNPTFLEV LEEFPSLHXT AAFLLSQLPI LKPRYYSISS    906
```

Figure 16B

```
DNOS    SPRRVSDEIH LTVAIVKYRC EDGQGDERYG VCSNYLSGLR ADDELFHFVR   1160
BENOS   APNAHPGEVH LTVAVLAYRT QDGLGPLHYG VCSTWLSQLK TGDPVPCFIR    996
RNNOS   SPDMYPDEVH LTVAIVSYHT RDGEGPVHHG VCSSWLNRIQ ADDVVPCFVR   1229
MMNOS   SQDHTPSEVH LTVAVVTYRT RDGQGPLHHG VCSTWIRNLK PQDPVPCFVR    956

                  ————NADPH·Ribose————
DNOS    SALGFHLPSD RSRPIILIGP GTGIAPFRSF WQEFQVLRDL DPTAKLPKMW   1210
BENOS   GAPSFRLPPD PYVPCILVGP GTGIAPFRGF WQE-RLHDIE SKGLQPHPMT   1045
RNNOS   GAPSFHLPRN PQVPCILVGP GTGIAPFRSF WQQ-RQFDIQ HKGMNPCPMV   1278
MMNOS   SVSGFQLPED PSQPCILIGP GTGIAPFRSF WQQ-RLHDSQ HKGLKGGRMS   1005

DNOS    LFFGCRNRDV D-LYAEEKAE LQKDQILDRV FLALSREQAI PKTYVQDLIE   1259
BENOS   LVFGCRCSQL DHLYRDEVQD AQERGVFGRV LTAFSREPDS PKTYVQDILR   1095
RNNOS   LVFGCRQSKI DHIYREETLQ AKNKGVFREL YTAYSREPDR PKKYVQDVLQ   1328
MMNOS   LVFGCRHPEE DHLYQEEMQE MVRKRVLFQV HTGYSRLPGK PKVYVQDILQ   1055

                  ————NADPH·Ade————
DNOS    QEF-DSLYQL IVQERGHIYV CGDVTMAEHV YQTIRKCIAG KEQKSEAEVE   1308
BENOS   TELAAEVHRV LCLERGHMFV CGDVTMATSV LQTVQRILAT EGDMELDEAG   1145
RNNOS   EQLAESVYRA LKEQGGHIYV CGDVTMAADV LKAIQRIMTQ QGKLSEEDAG   1378
MMNOS   KQLANEVLSV LHGEQGHLYI CGDVRHARDV ATTLKKLVAT KLNLSEEQVE   1105

DNOS    TFLLTLRDES RYHEDIFGIT LRTAEI---- --HTKSRATA RIRMAS----   1348
BENOS   DVIGVLRDQQ RYHEDIFGLT LRTQEVTSRI RTQSFSLQER HLRGAVPWAF   1195
RNNOS   VFISRLRDDN RYHEDIFGVT LRTYEVTNRL RSESIAFIEE SKKDADE-VF   1427
MMNOS   DYFFQLKSQK RYHEDIFGAV F-SYGA---- -KKGSALEEP --KAT-----   1142


                                                              1350
DNOS    --------QP                                            1205
BENOS   DPPGPDTPGP                                            1429
RNNOS   --------SS                                            1144
MMNOS   --------RL
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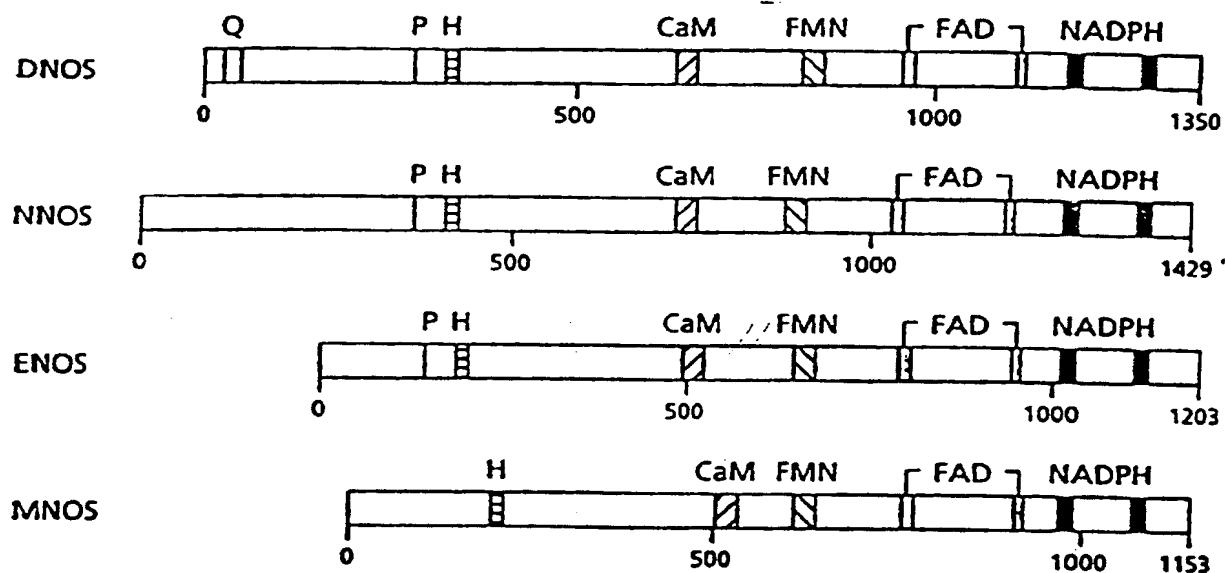
Figure 16C
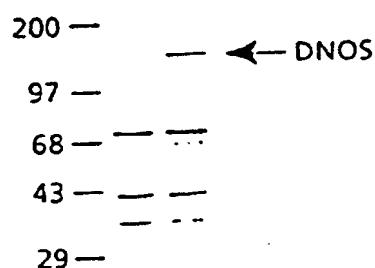
Figure 16D

Figure 17A

Figure 17B

H   B

9.5 —
7.5 —

4.4 —

2.4 —

1.4 —

MLC
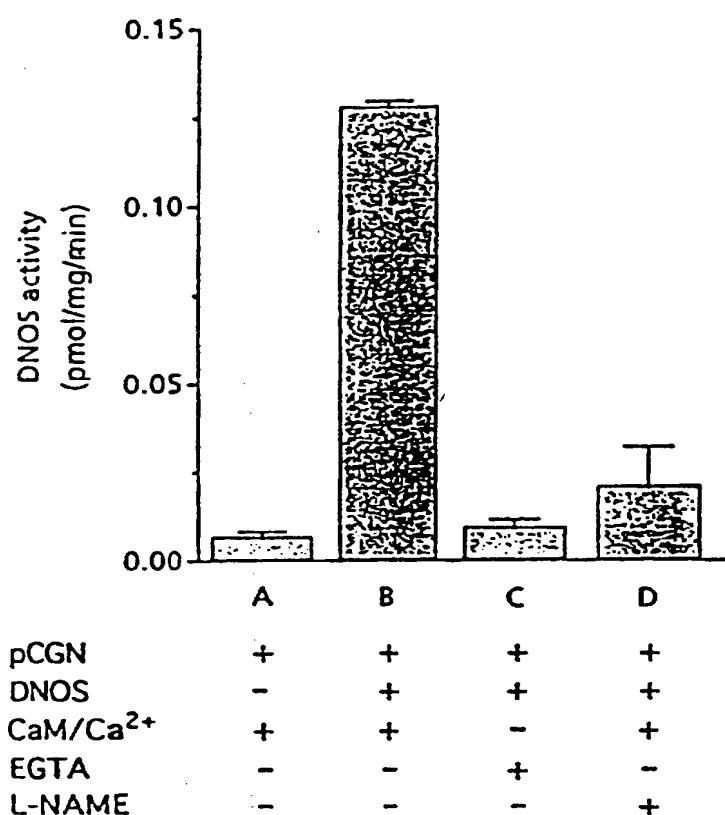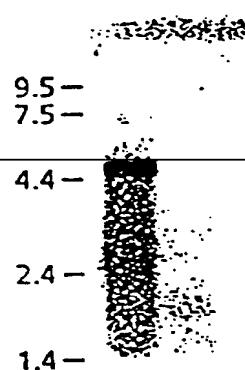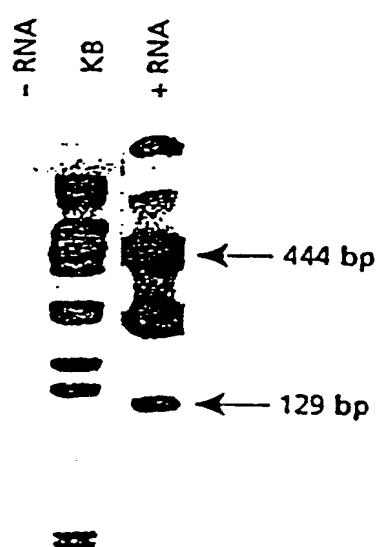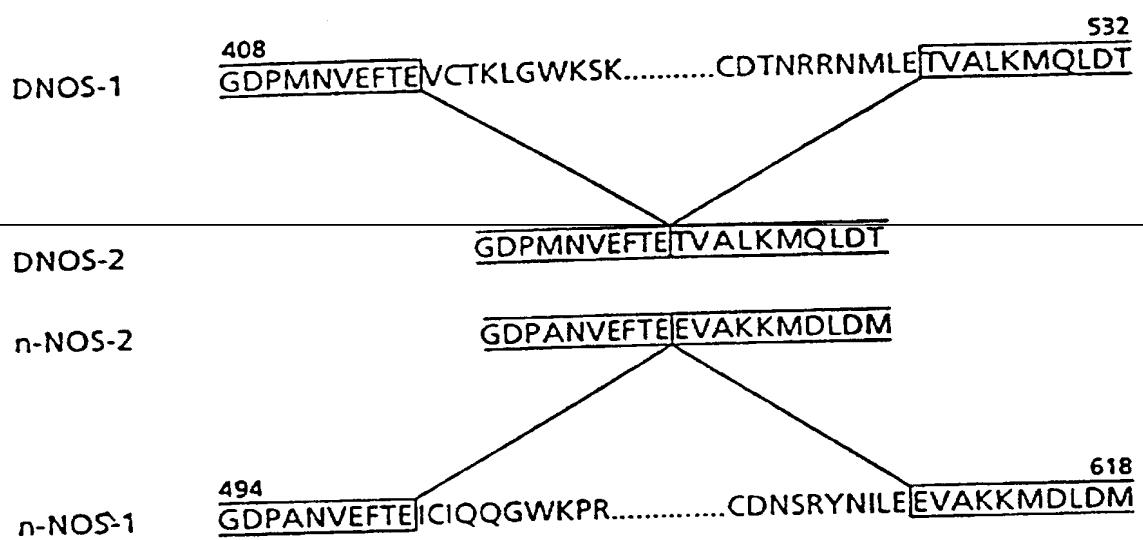
Figure 18A

Figure 18B

Figure 18C

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GAATTCCGTTTTTTGAAAAGTGAAGCAATTGAGTGCGGCCCGAAAAAGAGAGCCGCA
GAAAGTTTGCGAACAGAATTTAATCAAAAACTTGGAGGGTAAATTGTCCAAGTGGT
TCACCTGTTGGCTGCATTTTAAATCAACGAGGCAAACAATCAGCGCAGAGGAGCTG
CTCCACGTTCCCCGGACAAGATGTCGCAGCATTTCACATCGATATTTGAGAACCTGC
GATTCGTGACCATCAAACGTGCGACAAATGCGCAACAGCAACAGCAGCAGCAGCAG
CAACAGCAACTTCAGCAGCAGCAGCAGCAGCTGCAGCAACAGAAGGCACAGACAC
AGCAACAAAATAGCAGAAAAATCAAAACTCAAGCAACGCCAACGTTGAATGGCAAT
GGGCTCTTGAGCGGCAATCCAAATGGTCGGAGGCGGTCAACTCCTCGCCCAGCCATGA.
AGTGGACCATCCGGGTGGAGCACAAGGAGCTCAAGCAGCAGGAGGCTTGCCATCTT
TAAGTGGCACGCCATTGAGGCACCACAAGCGCGCCAGTATCTCCACAGCATCGCCTC
CAATTCGCGAACGGCGTGGCACCAACACCAGCATCGTGGTCGAACTGGATGGCAGT
GGCAGCGGGAGTGGGAGTGGCGGTGGTGGCGTTGGCGTTGGTCAGGGTGCGGGTTG
TCCTCCCTCGGGCAGCTGCACTGCGTCCGGAAAAAGTTCGCGGGAACTATCGCCGTC
GCCGAAAAACCAACAGCAGCCCAGAAAGATGTCACAGGATTATCGGTCGCGTGCCG
GCAGCTTTATGCACCTGGACGACGAGGGACGCAGTCTGCTGATGCGCAAGCCGATG
AGACTGAAGAACATCGAGGGCAGGCCGGAGGTCTACGACACGCTGCACTGCAAGGG
TCGCGAGATTCTTTCCTGCTCGAAGGCCACCTGTACGAGCAGCATTATGAACATTGG
CAATGCGGCGGTGGAGGCCAGGAAATCCGATCTGATCCTCGAACACGCCAAGGACT
TCCTCGAGCAGTACTTTACATCGATAAAGCGTACATCATGTACCGCCCACGAGACGC
GATGGAAACAGGTGCGCCAGAGCATTGAGACCACTGGACACTATCAGCTAACCGAA
ACGGAGCTAATTTATGGTGCCAAATTGGCCTGGCGCAATTCTTCACGTTGCATTGGC
CGAATACAATGGTCGAAGTTGCAGGTCTTTGACTGTCGTTATGTGACAACAACAAGT
GGCATGTTTGAAGCCATTTGCAATCACATTAAATATGCAACAAATAAGGGCAACCTG
AGATCGGCCATCACGATATTTCCACAACGCACAGATGCCAAGCATGATTATCGCATT
TGGAATAACCAATTAATATCTTATGCCGGCTACAAGCAGGCGGATGGAAAAATCAT
TGGCGATCCCATGAATGTGGAGTTTACAGAGGTCTGCACCAAGCTGGGCTGGAAGA
GCAAGGGCAGCGAGTGGGACATACTGCCATTGGTGGTCTCGGCCAATGGTCACGAT
CCGGACTACTTTGATTACCCGCCCGAATTGATACTGGAAGTTCCGCTGACCCATCCC
AAATTCGAATGGTTCTCGGATCTGGGACTGCGATGGTACGCCCTGCCCGCCGTATCC
AGTATGCTGTTCGATGTGGGCGGCATTCAGTTTACGGCCACCACATTCAGTGGTTGG
TACATGTCGACAGAGATTGGCAGCCGGAATTTATGCGACACAAATCGCCGCAATAT
GCTGGAGACGGTGGCGCTGAAGATGCAACTGGACACCCGTACGCCCACATCCTTGT
GGAAGGACAAGGCTGTGGTGGAGATGAACATTGCCGTGCTCCACTCCTACCAGAGT
CGCAACGTGACCATTGTGGATCACCACACGGCCAGCGAGAGCTTTATGAAGCATTTC
GAGAACGAGTCCAAGCTCAGGAATGGGTGTCCCGCTGATTGGATTTGGATCGTGCC
GCCGCTGTCGGGCTCCATAACGCCGGTATTCCATCAGGAGATGGCTCTGTACTACCT
GAAGCCCTCGTTCGAGTACCAGGATCCCGCCTGGCGAACCCACGTGTGGAAAAAGG
GGCGTGGCGAGAGCAAGGGCAAGAAGCCAAGACGTAAATTCAATTTTAAACAAATC
GCTAGGGCTGTGAAATTTACATCGAAACTATTTGGACGCGCCTTATCGAAACGCATA
AAGGCAACAGTTCTATATGCCACCGAAACTGGCAAATCGGAGCAGTATGCGAAGCA
ACTTTGTGAACTCCTAGGGCACGCATTCAATGCACAGATATATTGCATGTCCGACTA
CGATATATCCTCCATTGAGCACGAGGCATTGTTAATTGTTGTGGCCTCCACCTTTGGC
AACGGTGATCCCCCCGAAAACGGCGAGCTTTTCTCCCAGGAATTGTATGCGATGCGT
GTCCAGGAGTCTTCCGAGCATGGATTGCAGGACTCCAGCATTGGCTCGTCAAAGTCC
TTCATGAAGGCCAGCTCGCGGCAGGAGTTCATGAAGCTGCCACTGCAACAGGTGAA
GAGAATCGACCGATGGGACTCGCTGCGGGGCTCCACCTCGGACACCTTCACCGAGG
AGACCTTTGGTCCCCTCTCCAATGTCCGGTTTGCCGTTTTTGCCCTCGGCTCCTCGGC
CTATCCAAATTTCTGCGCCTTCGGTCAGTATGTGGACAACATTCTGGGCGAGCTGGG
CGGCGAACGCCTGCTGAGGGTGGCCTACGGCGACGAGATGTGCGGACAGGAGCAGT
CGTTCCGGAAGTGGGCGCCCGAGGTATTCAAGTTGGCCTGCGAGACCTTCTGCCTGG
ATCCAGAGGAGAGCCTTTCGGATGCCTCGCTAGCCCTGCAGAACGATTCGCTGACTG
TGAATACGGTGCGCCTGGTGCCGTCGGCGAATAAGGGATCCCTGGACAGCAGTTTAT
CCAAGTACCACAACAAGAAGGTGCACTGCTGCAAGGCGAAGGCGAAGCCCCACAAT
TTGACCCGTTTGAGTGAGGGAGCCAAGACAACGATGCTGCTGGAGATCTGTGCACCT
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Figure 19A

GGCTTGGAGTACGAGCCGGGTGATCATGTGGGCATCTTTCCGGCGAATCGAACGGA
ACTGGTCGACGGACTGCTAAATCGACTGGTGGGTGTGGATAATCCCGACGAGGTGC
TGCAGTTGCAATTGCTAAAGGAAAAGCAGACATCGAATGGTATATTCAAGTGCTGG
GAGCCGCACGACAAAATACCGCCGGATACTCTAAGGAATCTACTGGCCCGATTCTTT
GATCTGACCACTCCGCCATCGCGACAGCTACTCACCCTGCTGGCTGGATTCTGTGAG
GACACCGCGGACAAGGAGCGGCTGGAGTTGCTGGTCAACGATTCGTCGGCCTACGA
GGACTGGCGGCACTGGCGGCTGCCGCACCTGCTGGACGTCCTCGAGGAGTTCCCTTC
GTGCCGACCACCGGCTCCCCTTCTGCTTGCCCAACTAACGCCGCTGCAGCCTCGCTT
CTATTCCATTTCCTCGTCGCCGCGCCGCGTTAGTGACGAAATCCACCTGACGGTGGC
CATCGTGAAGTACCGTTGTGAAGATGGTCAGGGTGACGAGCGGTACGGCGTGTGCT
CTAACTATCTATCCGGCTTGCGGGCAGACGACGAGCTGTTCATGTTCGTGAGAAGCG
CCTTGGGCTTCCATTTGCCCAGCGATCGGAGTCGTCCCATTATTCTGATTGGTCCTGG
CACAGGAATAGCTCCATTCCGCTCCTTTTGGCAGGAGTTCCAGGTGCTACGCGACCT
TGATCCCACGGCCAAATTGCCCAAGATGTGGCTCTTCTTTGGCTGCCGGAATCGGGA
TGTGGACTTGTACGCCGAGGAGAAGGCAGAGCTACAGAAGGATCAAATCCTAGACC
GAGTTTTTCTCGCTCTGTCCAGGGAGCAGGCCATTCCGAAGACATATGTGCAGGACC
TGATTGAGCAGGAATTCGATTCGTTGTACCAGTTGATTGTCCAGGAGCGGGGCCACA
TCTACGTCTGCGGCGATGTCACAATGGCCGAGCATGTGTACCAGACCATCAGGAAGT
GCATTGCCGGCAAAGAGCAGAAAAGCGAGGCGGAAGTTGAGACATTTTTGCTAACA
CTGCGGGACGAAAGTCGCTACCACGAGGACATCTTTGGCATCACGCTGCGAACGGC
TGAGATACACACAAAGTCAAGGGCCACGGCCAGGATACGAATGGCCTCCCAGCCCT
AAGGATAGATATTCGAAGTAATCAAAATAGGAGGGTGACATATCCAAATTCGAGAG
GAATACCAAGCACTTGCTCTTTTTTTCTTCCATATTCAAATGCAATTAAATATTGTC
GCTTTGTTCATTACATATTCGTATGAATAACGTTTAAATAAATTACATTTTATTATTG
ATTCTAATGTACAAATCAATTGTGAAATCAAAATCTAAATGTTAAAATATATTTCAA
ATAAACGAATCGAAAAGGAATTC

Figure 19B

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/12   C12N15/53   C07K14/435   C12N9/02   A61K38/17
          A61K38/44   G01N33/68   //A01K67/027,C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C07K   C12N   G01N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DNA AND CELL BIOLOGY, (1993 SEP) 12 (7) 589-95, USUI, T. ET AL. 'Isolation of Drosophila CREB -B: a novel CRE-binding protein.'	26,27
A	see the whole document	18-28

X	MOLECULAR AND CELLULAR BIOLOGY, (1992 SEP) 12 (9) 4123-31, SMOLIK, S. ET AL. 'A cyclic AMP-responsive element-binding transcriptional activator in Drosophila melanogaster, dCREB-A, is a member of the leucine zipper family.' see the whole document	29

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[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 March 1996	2 6. 03. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

Form PCT-ISA/210 (second sheet) (July 1992)

Ir auonal Application No
PCJ/JIS 95/13198

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	23RD ANNUAL MEETING OF THE SOCIETY FOR NEUROSCIENCE, WASHINGTON, D.C., USA, NOVEMBER 7-12, 1993. SOCIETY FOR NEUROSCIENCE ABSTRACTS 19 (1-3). 1993. 1066, XP 000566270 TULLY, T. ET AL. 'Independent memories in Drosophila after Pavlovian conditioning.' see abstract	36
A	MOLECULAR ENDOCRINOLOGY, vol. 7, no. 2, February 1993 page 145-153 DE GROOT, R. & SASSONE-CORSI, P. 'Hormonal control of gene expression: multiplicity and versality of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators' cited in the application see figure 3	1-28
A	EUROPEAN JOURNAL OF NEUROSCIENCE, (1994 AUG 1) 6 (8) 1362-70, MULLER, U. 'Ca2+/calmodulin-dependent nitric oxide synthase in Apis mellifera and Drosophila melanogaster.' see the whole document	33-35
A	SCIENCE, vol. 265, 22 July 1994 US, pages 542-546, O'DELL, T. ET AL. 'endothelial NOS and the blockage of LTP by NOS inhibitors in mice lacking neuronal NOS' cited in the application see the whole document	33-36
A	EMBO JOURNAL, vol. 11, 1992 EYNSHAM, OXFORD GB, pages 1503-1512, RUPPERT, S. ET AL. 'Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes' cited in the application see figure 3	1,2,6,28
P,X	CELL, (1994 OCT 7) 79 (1) 49-58, YIN, J. ET AL. 'Induction of a dominant negative CREB transgene specifically blocks long - term memory in Drosophila.' see the whole document	1-28,36, 37

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CELL, (1995 APR 7) 81 (1) 107-15, YIN, J. ET AL. 'CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long - term memory in Drosophila.' see the whole document ---	1-28,36, 37
P,X	MOLECULAR AND CELLULAR BIOLOGY, (1995 SEP) 15 (9) 5123-30, YIN, J. ET AL. 'A Drosophila CREB /CREM homolog encodes multiple isoforms, including a cyclic AMP-dependent protein kinase-responsive transcriptional activator and antagonist.' see the whole document ---	1-28
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1995 SEP 26) 92 (20) 9072-6, REGULSKI, M. ET AL. 'Molecular and biochemical characterization of dNOS: a Drosophila Ca2+/calmodulin-dependent nitric oxide synthase.' see the whole document -----	33-35

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   **1-9,11-16**
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 1-9,11-16 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)